# Endocrine disruption and human health.

## From populations to cells: an integrated approach in the study of Bisphenol A.

Submitted by Riccardo Cipelli to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences
In March 2013



I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

## Abstract

*Background.* Endocrine disruptors (EDC) are exogenous compounds that mimic the action of natural hormones and alter the normal endocrine system. Life-long chronic exposure to Bisphenol A (BPA), a putative EDC, has been linked with risk of metabolic disorders in epidemiological studies.

*Objectives.* The aim was to study the human health effects of exposure to BPA, using an integrated approach combining environmental epidemiology and toxicology.

*Methods.* Urinary levels of BPA exposure were measured in participants of the InChianti longitudinal study, a representative population-based study of Italian adults, at the Baseline (1998-00) and nine years later (3rd Wave, 2007-09). Hormones levels and the gene expression of specific target genes were the end points considered. Results were validated in laboratory studies on a human leukemic T-cell line (Jurkat cells).

*Results.* In general, urinary BPA (uBPA) concentrations were higher among men and younger respondents, and within subjects uBPA concentrations were correlated ($r$=0.58; $p$=0.013, model adjusted for age, sex, urinary creatinine).

At baseline, uBPA concentration were associated with higher total testosterone concentrations in men (β = 0.05; 95% CI, 0.02–0.08). In the 3rd wave, gene expression analysis revealed positive associations between uBPA concentrations and ESR2 (estrogen receptor beta) expression (β=0.18; 95% CI: 0.04, 0.32) and ESRRA (estrogen related receptor alpha) expression (β= 0.17; 95% CI: 0.02, 0.32).

In a following *in vitro* study, BPA exposure (0.001-1 micro molar) led to enhanced expression of ESRRA and ESR2 in Jurkat cells over a period of 72 hours.

*Conclusions.* Results indicate that BPA is bioactive in the human body and is able to alter circulating hormone concentrations and estrogen receptor/estrogen-related receptr gene expression. In particular, given the role of ERRα as a major control point for

oxidative metabolism and heart development, this research provides indications on the possible molecular mechanisms of action of BPA in metabolic diseases.

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## Abbreviations

AR, androgen receptor

BPA, bisphenol A

uBPA, urinary bisphenol A

BEL, Brixham Environmental Laboratory

CDC, Centers for Disease Control and Prevention

CVD, cardiovascular disease

CAD, coronary artery disease

DES, diethylstilbestrol

E2:T, estradiol:testosterone ratio

EDC, endocrine disruptor compounds

EFSA, European Food Safety Authority

ER, estrogen receptors

ERα, *ESR1*, estrogen receptor alpha

ERβ, *ESR2*, estrogen receptor beta

ERRα, *ESRRA*, estrogen related receptor alpha

ERRβ, *ESRRB*, estrogen related receptor beta

ERRγ, *ESRRG*, estrogen related receptor gamma

FSH, follicle-stimulating hormone

GLP, good laboratory practice

GPR30, G-protein-coupled receptor

HPLC-MS/MS, high-performance liquid chromatography - tandem mass spectrometry

NCHS, National Center for Health Statistics

NHANES, National Health and Nutrition Examination Survey

NIEHS, National Institute of Environmental Health Sciences

NR3B, orphan nuclear receptors subfamily

NTP-CERHR, National Toxicology Program- Center for the Evaluation of Risks to Human Reproduction

OR, odds ratio

PPARγ, peroxysome proliferator-activated receptor gamma

SERM, selective estrogen receptor modulator

SG, urine specific gravity

TDI, Tolerable Daily Intake

## Acknowledgements

First of all, I want to thank my supervisor, Professor Tamara Galloway. This has been undoubtedly the most intense and demanding experience of my life and she has always been a reference for me in bad and good times. I will always be grateful to her for the incredible opportunity she gave me to take part to this project and be part of a team which I am sure I will not find anywhere else.

I am therefore grateful to all the other members of the group: Professor David Melzer, Dr. Lorna Harries and Dr. Ed Keedwell from whom I have learnt all the aspects of this research and most importantly, the importance of team work.

I want to thank all the friends and colleagues I have met here at Exeter. Olivia, Aisha, Paul, Shelly, Julia, Francesco, Luigi… the full list would be too long but I thank everyone who made working in here such a unique experience.

This dissertation, my little piece of endeavour, is dedicated to my mum, who I miss every day, and dad. Simply, as I sad once, *vi devo tutto*.

Most of all, I am not able to find the words to thank you, Giulietta, love of my life. I will always be indebted with you for your patience, your faith in me…and for our little boy, Filippo, Thank you.

# 1. Introduction

*1.1 Endocrine Disruptors Compounds (EDC).*

The term Endocrine Disruptors Compounds (EDC) was firstly agreed in 1991 by a group of scientist who gathered at the U.S. Wingspread Conference to discuss emerging chemically-induced alterations in wildlife populations (Colborn et al. 1993). Evidence brought to the meeting suggested that a large number of man-made chemicals, used in a variety of industrial and agricultural applications and then released in the environment, could mimic the action of endogenous hormones and have the potential to alter the endocrine system of animals.

In particular, hormone-like compounds were discussed which were able to bind to hormone nuclear receptors such as the estrogen receptors, androgen receptor, and thyroid hormone receptor. Once bound, these so-called EDCs can have an agonist or antagonist action on the receptor activity (Swedenborg et al. 2009).

The effects observed in wildlife animals differed from traditional toxicology endpoints which normally focussed on gross changes in morphology, development, mortality, or tumor incidence. Early evidence showed that hormone-like compounds could elicit a more subtle toxicity and affect sexual differentiation, reproductive function, neurobehavioral development, and induce autoimmune diseases (Vandenberg et al. 2009).

Most notably, these effects were mainly seen in the offspring of exposed animals rather than in the adults (Colborn et al. 1993). Observations on wildlife animals shared similarities with studies on the pharmacological agent diethylstilbestrol (DES), a potent synthetic estrogen administered to pregnant women from 1948–71 (Colborn et al. 1996). Whereas women exposed to DES during pregnancy were relatively unharmed, their daughters who were exposed *in utero* (so-called DES daughters) had significantly

increased rates of uterine, cervical, and vaginal malformations. Due to these first observations, it was hypothesised that EDC exposure should be investigated in particular during critical periods of development such as perinatal and *in utero* exposure.

Moreover, hormone-like compounds were shown to contradict another dogma of toxicology: the linear relationship between dose and response. Multiple studies on EDCs have shown that the mechanism of EDC action is better described by a non-monotonic curve: biphasic dose response has been observed in many different endpoints (Vandenberg et al. 2012). As a result, EDC low dose effects are not necessarily predicted by high dose exposure studies. This created a lot of controversy, scientists suggested that the linear models routinely used for risk assessment purposes by government agencies should be rejected and replaced entirely in the study of EDC (Vandenberg et al. 2009). Also, given that making predictions about the effects of low doses by testing higher doses is not appropriate, the identification of relevant or environmentally relevant exposure in laboratory studies became a source of continuous debate.

Since the first hypotheses formulated in the 1990s, the study of EDC has evolved and included hundreds of compounds, and so has its definition, in the attempt to encompass an ever increasing and controversial field of research:

*"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"(W.H.O. 2002)*

Despite significant advances in the study of endocrine disruptors in the last two decades, this remains a controversial field and therefore in need of more study.

The initial focus on developmental effects and the importance of perinatal exposure has widened in recent years and concerns over adult exposure have grown. The incident of Seveso, Italy, in 1976 (Baccarelli et al. 2004) showed how exposure to dioxin could affect not just exposed infants or pre-natal fetuses but also adolescents and adults.

Moreover, recent laboratory and animal studies have shown that a variety of environmental EDCs, the so called "obesogens", can influence human adipogenesis and obesity (Grun and Blumberg 2006; Newbold et al. 2007). This may also lead to other metabolic syndromes such as type-2 diabetes. The resulting metabolic disruption is not necessarily linked to a prenatal exposure, rather to a low chronic lifelong exposure.

Similarly, studies on oral hormone female contraceptives build on the concern over the risk of adult exposure to hormone-like compounds and in particular to estrogenic compounds. Initial reports associated the use of oral contraceptives in women with breast cancer diagnoses (Calle et al. 1996), ovarian cancer, cervical cancer, and cerebrovascular disease (Beral et al. 1999). Another study found higher risk of myocardial infarction in women taking oral contraceptives (Tanis et al. 2001). Although some of the initial findings have been disputed (Hannaford et al. 2010), this remains extremely indicative of the risk of adult exposure to estrogenic compounds.

In such a controversial field of research, the need to combine different expertise in the study of EDC and adopt an integrated approach is crucial. Laboratory studies alone have not reached conclusive results. The convergence of environmental toxicology and molecular epidemiology is necessary to study and predict effects of environmental chemicals on human health (Jaffery et al. 2002).

The integration with environmental toxicology can provide epidemiology with biomarkers and molecular end points needed to assess the effects of environmental exposures on health and disease. On the other hand, understanding the mechanism of action and the toxic effects of a contaminant through toxicological studies is necessary to support scientific conclusions from observational studies.

### *1.2.1 Bisphenol A: commercial uses, human exposure, and biomonitoring studies.*

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is a chemical first synthesized in 1891 by condensation of two phenol groups and one acetone molecule. Dodds and Lawson (1936) were the first to discover BPA's estrogenic properties while in pursuit of a synthetic estrogen compound for pharmaceutical use. The two researchers later discovered the powerful estrogenic substance diethylstilbestrol (DES), and the research into BPA as a drug was put aside (Dickens 1975). In the late 1940s, chemists realized BPA's potential applications in industry; since then, BPA has been widely used as a monomer and plasticizer and is considered one of the world's highest production volume chemicals reaching in 2008 the amount of 5.2 million tons (Geens et al. 2012).
BPA finds application in a wide variety of domestic products containing polycarbonate or epoxy resins. Polycarbonate resins represent the main share of the BPA market and are largely used for packaging application. Products such as large refillable water bottles (commonly present in workplaces), sports bottles, baby bottles, home food containers and flatware contain polycarbonate resins. Epoxy resins are the second largest end use for BPA and, as inner coatings of food and beverage cans, help to protect metals from rusting and corrosion. Other uses of BPA include the production of flame retardants, thermal paper, polyester resins, and polyvinyl chloride (PVC) stabilizers used in water main pipes (Bailey and Hoekstra 2011).

BPA can migrate from containers and come into contact with the surrounding material, mainly because of two different processes: under normal conditions, residuals of the BPA manufacturing process, namely incomplete polymerization, can leach from the product; alternatively, BPA chemical bonds can be hydrolyzed releasing free BPA after brushing, under heat exposure or at an alkaline pH (Kang et al. 2003). It has been shown that BPA leaches from baby bottles, drinking and sport bottles, plastic stretch film, food cans, and dental sealant (Bailey and Hoekstra 2011; Brede et al. 2003; Brotons et al. 1995; Kawamura et al. 1999; Olea et al. 1996; Schecter et al. 2010). Until recently, ingestion was considered the main route of BPA human exposure (Vandenberg et al. 2007). Yet, new evidence suggests that exposure through inhalation (e.g. dust) and transdermal contact may have more than a negligible contribution to the total body burden (Geens et al. 2011; Geens et al. 2012).

In the last decade, biomonitoring studies have determined the extent of human exposure to BPA, particularly in industrialized nations. Detectable levels of BPA have been measured mostly in urine and blood/serum samples but also in amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood (reviewed in Vandenberg et al. 2007).

Urine is generally the matrix of choice for biomonitoring studies (Calafat 2011): evidences from toxicokinetic studies on non-human primates and humans suggest that almost the entire proportion of ingested BPA is excreted through urine (Doerge and Fisher 2011; Taylor et al. 2011). There is little evidence of BPA bioaccumulation in the body: BPA metabolism is in fact considered rapid (<24 hours). Once ingested, BPA is extensively absorbed from the gastrointestinal tract, converted into a glucuronide conjugate, and excreted via the kidneys. Similarly, inhaled and adsorbed BPA is excreted through urine but may not undergo such an effective metabolism and remain unconjugated (Pottenger et al. 2000). Yet, both conjugated (metabolized) and

unconjugated (free) BPA are measured in urine as urinary concentrations of total (free + conjugated) BPA.

Results from biomonitoring studies indicate an almost ubiquitous human exposure, with BPA being detected in the urine of more than 90% of subjects in surveys representative of the US (Calafat et al. 2008) and Canadian populations (Bushnik et al. 2010). Similar or even higher detection rates have been reported in smaller or less representative studies carried out in the US or Europe (Cobellis et al. 2009; Galloway et al. 2010; Kasper-Sonnenberg et al. 2012; Koch et al. 2012; Mahalingaiah et al. 2008; Meeker et al. 2010a; Mendiola et al. 2010; Pirard et al. 2012). Biomonitoring analyses carried out in China and Korea reported detectable levels of BPA in 50-75% of the subjects enrolled in the studies (He et al. 2009; Hong et al. 2009). However, in a follow-up study carried out in seven Asian countries, including China and Korea, BPA was detected in more than 90% of the samples (Zhang et al. 2011).

Internal BPA dose in humans can be estimated also by measuring BPA concentration in blood. Contrary to urine analysis, blood analyses normally measure free BPA concentrations.

Vandenberg et al (2010a) reviewed the literature of biomonitoring studies and identified the range of total urinary BPA concentrations (1-10 ng of BPA/ml urine, mean range), and unconjugated (free) BPA concentration (0.5–2.5 ng/ml).

### *1.2.2 Bisphenol A: experimental studies.* In vitro *and* in vivo *evidence of estrogenicity.*

Despite the early evidence (Dodds and Lawson 1936), BPA was assumed to be relatively harmless to human health and it was deemed safe for commercial use: BPA estrogenicity was not considered potentially harmful until the study of EDCs took off in the ‘90s (Vogel 2009). Since then, BPA has been extensively studied.

In 1988, the U.S. Environmental Protection Agency (EPA) established the BPA human Tolerable Daily Intake (TDI) using data from carcinogenesis studies on rodents. The lowest dose used in the studies was divided by an uncertainty factor of 1000 to determine the limit of 50 µg BPA/kg of body weight per day (50 µg/kg/day), considered the reference safety dose (Vogel 2009). The European Food Safety Authority (EFSA) set the same TDI in 2006 (EFSA Panel on Food additives 2006).

Like the estrogen hormones, BPA binds to the estrogen receptors (ER). In biochemical assays, BPA binds both estrogen receptor alpha (ERα) and beta (ERβ), with approximately 10 times higher affinity for ERβ (Kuiper et al. 1998; Matthews et al. 2001). Still, initially BPA was considered a weak environmental estrogen because its efficacy in binding ER is 1.000/10.000-fold less compared with 17β-estradiol, the predominant female estrogen hormone (Nagel et al. 1997).

In the last decade, a high number of experimental studies have questioned the assumption of BPA weak estrogenicity. Laboratory animal studies have shown how BPA can be physiologically active and cause adverse reproductive and developmental effects at low doses of exposure [Reviewed in: Chapin et al. (2008); Richter et al. (2007a); Thayer and Belcher (2011); Wetherill et al. (2007)].

Low dose exposure was initially defined as levels below the TDI (50 µg/kg/day) for *in vivo* animal studies, and below 100 nM BPA concentration for *in vitro* cell or organ culture studies (Wetherill et al. 2007). In light of the indications from biomonitoring studies (described in section 1.1.1), the range for low dose exposure experimental studies is now considered 1 µg/kg/day for *in vivo* animal studies, and 1-10nM BPA concentration for *in vitro* cell or organ culture studies.

The vast interest in this topic is testified by the full list of experimental studies on low dose effects of BPA in animals which, in 2005, counted more than 150 peer-reviewed

studies (vom Saal and Hughes 2005). Similarly, a few years later a panel of experts from the U.S National Toxicology Program (NTP-CERHR), in an attempt to assess the entire literature on BPA, included more than 550 studies in their review (Chapin et al. 2008).

Initially, *in vitro* studies showed that BPA is not merely a weak estrogen mimic. BPA can interact differently with the estrogen receptors compared to 17β-estradiol (Gould et al. 1998), and the different binding in the ligand-domain can cause a different recruitment of coactivators required for normal gene expression (Hall and Korach 2002; Pennie et al. 1998; Routledge et al. 2000). These initial findings prompted the classification of BPA as selective estrogen receptor modulator (SERM) like some pharmacological compounds.

However, other *in vitro* molecular studies have shown that BPA can interact with a number of other nuclear receptor systems, not just the ER. BPA has been reported to bind to the androgen receptor (AR) showing an anti-androgenic activity (Lee et al. 2003; Xu et al. 2005). Another study reported an opposite, agonist, effect on AR suggesting that BPA action can be tissue-specific (Richter et al. 2007b). Moreover, other studies have shown BPA to bind to the thyroid hormone receptor (Moriyama et al. 2002); the aryl hydrocarbon receptor (Bonefeld-Jørgensen et al. 2007); peroxysome proliferator-activated receptor gamma (PPARγ) (Kwintkiewicz et al. 2010); BPA was also reported to bind to G-protein-coupled receptor (GPR30) (Thomas and Dong 2006). In addition to nuclear hormone receptor activity, BPA can interact with non-classic ER systems: BPA shows a high binding affinity to the estrogen-related receptor-γ (ERRγ) in biomolecular studies (Okada et al. 2008; Takayanagi et al. 2006). ERRγ belongs to the orphan nuclear receptors subfamily NR3B, which include the three isoforms ERRα,

ERRβ and ERRγ. No previous studies have indicated that BPA interacts with the other two components of the orphan receptor family, ERRα and ERRβ.

In addition to these studies, it has been suggested that BPA could elicit its activity beyond the traditional nuclear receptor binding activity. A non-genomic mechanism has been suggested: BPA can have an impact on cellular physiology through rapid signalling mechanisms that are independent of nuclear hormone receptor activity that influences later gene expression (Nadal et al. 2000). These effects occur within minutes or seconds of exposure and are initiated by membrane-associated receptor systems that act to modulate the intracellular signalling (Alonso-Magdalena et al. 2005; Ropero et al. 2006; Soriano et al. 2012). Yet, these rapid signalling can later interact with the traditional nuclear receptor pathways and trigger a genomic response.

Investigation into *in vitro* molecular BPA action has been paralleled by the observation of physiological effect in animal studies and in particular in rodents (reviewed in (Chapin et al. 2008; Richter et al. 2007a). Studies on rodents have mainly focused on developmental effects after early *in utero* exposure. The literature in this sense is broad and covers studies on different endpoints: behavioural changes, and in particular aggression; learning and social interaction; developmental effects on the female reproductive system, timing of puberty and mammary gland development; developmental effects on the male reproductive system, and in particular sperm viability and reproduction (Richter et al. 2007a).

Other endpoints considered have been breast cancer incidence and effects on immune system but no clear evidence has emerged (Vandenberg et al. 2009).

In addition, other studies looked at BPA exposure and hormones levels. BPA has been linked with changes in levels of thyroid hormones in rodents, even though results were

inconsistent across studies (Wetherill et al. 2007). Similarly, studies on testosterone levels in rodents exposed to BPA showed both negative (Akingbemi et al. 2004), and positive (Ramos et al. 2003) correlation. Exposure to BPA has also been linked with effects on metabolism: decreased levels of adiponectin (Hugo et al. 2008); fat tissue accumulation (Marmugi et al. 2012); insulin resistance, pancreatic disruption and possible onset of diabetes (Alonso-Magdalena et al. 2006; Alonso-Magdalena et al. 2010; Ropero et al. 2008).

*1.2.3 Bisphenol A: observational studies. Epidemiological evidence and study limitations.*

As discussed above, the literature on laboratory *in vitro* and *in vivo* BPA studies is extensive. Indications of reproductive and developmental effects at low levels of exposure prompted requests to re-assess the standards for BPA safety and revise the TDI limit (Vogel 2009; vom Saal and Hughes 2005).

Yet, the evidence brought by experimental studies were not considered conclusive nor enough consistent (Chapin et al. 2008; EFSA Panel on Food additives 2006; Melnick et al. 2002). In general, the range of doses tested was criticised. Very low dose studies were criticised for susceptibility to a range of possible confounders or factors of variability that would cause a lack of robustness in the results: sample size, dose range, statistical analyses and experimental design received criticisms (Chapin et al. 2008; Haseman et al. 2001).

Moreover, results on rodent studies have been debated for their applicability to human risk assessment: the fact that the main BPA excretion route in rodents is through biliary excretion, and not urine as in humans, is considered a limitation of the results.

Additional elements of controversy and scrutiny were the way BPA was administered to caged animals (injected or orally absorbed) and, from a regulatory point of view, the use of "good laboratory practice (GLP)" protocols in the research (vom Saal et al. 2012).

After revising the entire literature, NTP-CERHR considered that there were "*some concern for effects on the brain, behaviour and prostate gland in foetuses, infants and children at current human exposures to BPA"* and that "*the possibility that Bisphenol A may alter human development cannot be dismissed"* (Chapin et al. 2008). Also, in 2007 the U.S. National Institute of Environmental Health Sciences (NIEHS) sponsored a meeting with an independent panel of Bisphenol A experts (vom Saal et al. 2007) that, among other recommendations, concluded:

*"There is a need for epidemiological studies relating health outcomes to BPA exposure, particularly during sensitive periods in development. These studies should be based on hypotheses from findings in experimental animals. This will require additional development of appropriate biomarkers in animal studies that can be used in epidemiological research."*

Epidemiological studies on the effects of exposure to BPA were very sparse until recently. In a recent review, Braun et al. (2011a) have counted 23 peer-reviewed scientific publications on BPA and human health; when the authors applied their inclusion/exclusion criteria, based on minimum requirements for study quality (sample sizes, correct selection and description of control or comparison groups, appropriate statistical analyses), only 10 studies were considered relevant.

Table 1.1 summarize the full list of epidemiology studies published between January 2008 and December 2012: few studies were published before 2008, generally these were based on a very limited number of observations, and issues have been raised relatively to the quality of urinary BPA measurements before the development of a standardized procedure in 2007 (Calafat 2011; Calafat et al. 2008).

As indicated in sections 1.1 of this introduction, the initial studies on EDCs focussed on the so-called sensitive window of exposure. For this reason, a number of epidemiological studies focused on the association of urinary BPA concentrations with developmental outcomes after *in utero* or childhood exposure.

Among those, most notably Braun et al. (2009) found that prenatal BPA exposure may be associated with externalizing behaviours in 2-year-old female. After gathering more data from the same prospective birth cohort study, the same researchers found that gestational BPA exposure affected behavioural and emotional regulation domains at 3 years of age, especially among girls (Braun et al. 2011b). These observational studies confirm previous indications from experimental studies where gestational BPA exposure was associated with disrupted neurodevelopment in animals. Overall, the study by Braun and colleagues could rely on different measurements of both mother and child exposure to BPA over a period of 2 years. As I will discuss later in the introduction, this represent an important point in the studies of human BPA exposure.

Another study investigated if peripubertal BPA exposure in girls could have an effect on breast and pubertal development but found no significant results (Wolff et al. 2010). Given the high number of participants (n=1151) and the high quality uBPA analysis, it would appear that peripubertal period could not be the only critical window of exposure for pubertal development. Similarly, other hypotheses emerging from experimental studies were investigated: sexual dysfunction, semen quality and risk of breast cancer all received attention (Table 1.1) but reached mixed or little conclusions.

Few studies investigated the effects of BPA exposure with semen quality in men and reached different conclusions. Meeker et al. (2010; 2011) found slightly lower levels, though not statistically significant, of sperm concentration, motility, and morphology in men (n=190) with high levels of uBPA from an infertility clinic. Because of the

relatively low number of patients, relationship between BPA and sperm DNA damage could warrants further investigation. An occupational study on BPA exposure found much more convincing evidence of an adverse effect of BPA on semen quality (Li et al. 2011). They compared 218 men with and without BPA exposure in the workplace and found more than three times the risk of lowered sperm concentration and lower sperm vitality. The study design was cross-sectional and, as discussed later in the introduction, this represents a limitation. On the other hand, Mendiola et al. (2010) measured uBPA concentrations in partners of pregnant women in four U.S. cities. Unsurprisingly, they found that uBPA concentrations were not associated with reproductive function and sperm count. The fact that only fertile men were selected represents a limitation for the conclusions of this study.

In recent years, the U.S. National Health and Nutrition Examination Survey (NHANES) has emerged as a valuable platform for epidemiological analysis on BPA exposure.

The NHANES is the American food consumption database program conducted by the National Center for Health Statistics (NCHS) and the Centers for Disease Control and Prevention (CDC). NHANES surveys are cross-sectional and contain a core set of physical examinations, clinical and laboratory tests, and personal interviews. Since 2003, the design also includes the testing of uBPA concentrations in a subset of participants. The great number of participants in these surveys is certainly an important factor for the interpretations of the results.

Using the data from NHANES, Meeker and Ferguson (2011) showed an inverse association with altered thyroid hormones in adults and adolescents. As discussed in section 1.2.2, these results support previous experimental findings.

Recent research has reported the investigation of metabolic disruption in adults exposed to BPA. An increasing number of studies have associated levels of BPA exposure with

the incidence of metabolic syndromes, which include obesity, type-2 diabetes, heart disease and hypertension.

A great deal of interest has been raised by the analysis of data from the NHANES that reported associations of BPA exposure with self-reported diagnosis of cardiovascular disease (Odds Ratio, OR, per 1-SD increase in BPA concentration: 1.4, 95% CI 1.2–1.6) and diabetes (OR 1.4, 95% CI 1.2–1.6) in a group of adults (aged 20 through 74) examined in 2003/04 (Lang et al. 2008). In addition, BPA concentrations were associated with clinically abnormal concentrations of the liver enzymes y-glutamyltransferase (OR 1.3, 95% CI 1.1-1.5) and alkaline phosphatase (OR 1.5, 95% CI 1.2-1.8).

The association with cardiovascular disease was confirmed in the following survey (NHANES 2005/06) in which an entirely new set of participant was enrolled (Melzer et al. 2010). Similar to the first study, odds ratio for cardiovascular disease was 1.33 (95% CI 1.01- 1.75). Associations with diabetes did not reach significance in 2005/06, but pooled estimates (2003/04 and 2005/06) remained significant (OR 1.24, 95% CI 1.1-1.4), similarly with the levels of liver enzymes.

In both studies the number of participants was elevated (n=1455 in 2003/04 and n=1493 in 2005/06) to provide sufficient statistical power to detect low-dose effects; the authors reported 80% power in detecting an OR of 1.4 (Melzer et al. 2010), and an extensive array of sensitivity analyses was performed on the data. Another strong point of the studies was the population representativeness of the study sample and the high standard and accuracy of analysis recognised in the NHANES surveys.

Despite the high standard of the analyses and the indication of repeated findings, the evidence brought by the two epidemiological studies was not considered conclusive. In

particular, a set of specific limitations in the two studies was identified (Braun et al. 2011a; EFSA Panel on Food Contact Materials 2010; Hengstler et al. 2011; Sharpe 2010).

First, both studies were based on cross-sectional designs and this was considered limiting the interpretability of the studies. Due to the study design no indication on causation could be drawn. It was theoretically possible, for example, a reverse interpretation of the results: suffering an event of coronary artery disease could lead to a change in dietary habits, hence a change in exposure to BPA (Sharpe 2010).

The second limitation, more importantly, was that in the NHANES surveys uBPA concentrations were measured only after the time of CVD and diabetes diagnoses. Given the long latency periods of the outcomes considered, it was pointed out that more indications were needed on the stability of BPA exposure during the etiological window of development for such diseases (Braun et al. 2011a; Hengstler et al. 2011). In other words, indications whether a single BPA measurement could be representative of a long term exposure or not were needed. In addition to this, another source of uncertainty over the estimation of BPA exposure was that, in the NHANES surveys, uBPA was measured in single-spot urine specimens. It has been show that values of uBPA concentration may vary within the day if measured in single-spot samples (Arakawa et al. 2004; Teeguarden et al. 2011; Ye et al. 2011). Within day variability may depend on two factors: urine volume production and a variable BPA intake. Variable urine production can be adjusted in epidemiological analysis using measure of creatinine excretion (Calafat et al. 2005) or urine specific gravity (SG) (Mahalingaiah et al. 2008), although even these approaches could present some limitations (Barr et al. 2005). On the other hand, a variable BPA intake can produce a spike in urine concentration difficult to account for. For this reason, 24-hour urine collection has been recommended

in epidemiology studies because it would account for within day variability. Yet, longer term measures remain needed in repeated specimens over ideally months and years.

Third, a risk of misclassification was indicated also for the self-reported diagnoses on pre-existing cardiovascular disease and diabetes. It was pointed out that, ideally, a medical examination would be more precise than the questionnaire-based survey in order to reduce the risk of misclassification (Hengstler et al. 2011).

Finally, BPA bioactivity in the human body at physiological levels was questioned. According to some authors and regulatory agencies, the environmental level of human exposure would not permit BPA binding to nuclear receptors and to trigger any molecular reaction (EFSA Panel on Food Contact Materials 2010). This is based on the consideration that orally absorbed BPA is subject to great first pass metabolism and BPA weak affinity for ERs.

The metabolism of BPA in the human body and the difference between conjugated and unconjugated BPA have been introduced previously when discussing biomonitoring studies (section 1.1.1). Once entered the body, only free BPA is considered capable of estrogenic activity. Glucuronide conjugate BPA has little or no affinity for the ERs (Matthews et al. 2001). There are two toxicokinetic studies indicating that BPA is rapidly metabolized and excreted, therefore suggesting that BPA is only present in the body as glucuronide conjugate (Völkel et al. 2002; Völkel et al. 2005), and these studies received a lot of attention from regulatory agencies (Vandenberg et al. 2010b).

On the other hand, evidence from biomonitoring studies, where free BPA was measured in blood samples (as discussed in section 1.1.1), contrast the conclusions reached by Volkel and colleagues. These conclusions have been heavily criticised for different flaws in the study design (Vandenberg et al. 2010b). Therefore, BPA bioactivity in the human body is considered a controversial topic and in need of more study.

*1.3 Aim of the research.*

The purpose of my research was to join a toxicological approach with observational human studies to examining the effects of low dose chronic exposure to BPA. Given all the indications emerged from regulatory agencies, I set out to investigate the major points of the debate around BPA toxicity and the limitations that could be addressed through my research.

In order to do that, I took advantage of the information supplied by a population-based longitudinal study, the InChianti study. InChianti is a prospective population-based survey, designed by the laboratory of Clinical Epidemiology of the Italian National Research Council of Aging (INRCA, Florence, Italy) and carried out in the Chianti geographic area of Tuscany, Italy (Ferrucci et al. 2000). This database includes data from 1,453 participants (age range 20–102 years) randomly selected from two municipalities: Greve in Chianti and Bagno a Ripoli. Data collection started in September 1998. Participants were then contacted every three years (2001-03; 2004-06; 2007-09) for follow-up assessment (see Figure 1.1).

At the study baseline, participants responded to an extensive initial home interview and underwent a complete medical examination. Participants were also requested to collect 24-hour urine, avoiding any meat and fish consumption during the day and over the previous 48 hours. Blood samples were collected on the same day. Clinical information was then obtained by routine laboratory analyses of blood and urine samples. This included levels of inflammatory proteins; sex hormones levels; liver enzymes and lipid levels; glucose metabolism; and a collection of RNA specimens. During the successive waves of the study, information was collected following a similar methodology but for

the urine samples that were collected as single-spot in the morning.

For this study, uBPA concentrations were measured in a subset of the urine samples collected at Baseline (1998-00) and the 3rd Wave (2007-09). Aliquots of urine samples were shipped from Italy and analyzed by the Brixham Environmental Laboratory (BEL) (Devon, UK), using state-of-the-technology high-performance liquid chromatography-isotope dilution tandem mass spectrometry (HPLC-MS/MS), and the analytical method adopted by the CDC (Calafat et al. 2008).

Objectives of the research (summarized in Figure 1.2):

1. To estimate the level of BPA exposure in a large-scale European representative population. *(Chapter 2)*
2. To investigate associations between uBPA concentration and levels of sex hormones in the study participants. Given the indications from experimental studies, the study hypothesis was that BPA exposure could alter reproductive hormone concentrations measured in serum samples. High quality analysis of 24-hr uBPA concentrations account for the controversy over BPA and within-day variability. *(Chapter 2)*
3. In addition, RNA specimens collected during the 3rd Wave were used in gene expression analysis. A set of specific target genes was identified and levels of gene expression were analysed for association with uBPA concentration. The study hypothesis was that BPA *in vivo* bioactivity could be measured through gene expression analysis of the nuclear receptors activity in blood cells. *(Chapter 3)*
4. Following up what emerged from the *in vivo* analysis, I set out to investigate

BPA bioactivity in an *in vitro* study on a human leukaemic T-cell line (Jurkat cells). In this study, I investigated the mechanisms and timing of activation of the target genes and explored BPA ability to modulate their expression in a time window of 72 hours. *(Chapter 4)*

5. I will also present the results of uBPA measured in single-spot urine samples collected during the 3rd Wave of InChianti in 2007-09. I have explored the association between uBPA concentrations measured in samples collected during the 3rd Wave and nine years earlier, at Baseline. I provide indication of variability in uBPA measurement over a long period of time in light of the controversies over BPA epidemiological findings. I could also draw a comparison between uBPA concentrations measured in 24-hours and single-spot samples and therefore I will comment on the validity of the two measurements. *(Chapter 5)*

Table 1.1 Summary of epidemiological studies examining the association of BPA with human health outcomes (2008-2012). [uBPA] = Urinary BPA concentrations.

| **Endpoint investigated**<br>**Reference Study** | **Design** | ***N*** | **Description** |
|---|---|---|---|
| *Developmental effects* | | | |
| Braun et al. (2009; 2011b) | Prospective cohort | 249; 244 | BPA exposure during pregnancy, but not childhood, was associated with externalizing behavior in 2/3 yrs girls and confirmed in follow-up study. |
| Miao et al. (2011) | Cross sectional | 153 | Occupational exposure to BPA during pregnancy was associated with shortened anogenital distance in male offspring. |
| Padmanabhan et al. (2008) | Cross sectional | 40 | No association between [uBPA] and birth outcomes. |
| Wolff et al. (2008a) | Cross sectional | 192 | No association between [uBPA] and pubertal development. |
| Wolff et al. (2008b) | Prospective cohort | 367 | No association between [uBPA] and most birth outcomes. |
| Wolff et al. (2010) | Prospective cohort | 1151 | No association between [uBPA] and breast/pubertal development. |
| *Hormones levels* | | | |
| Galloway et al. (2010) | Cross sectional | 715 | [uBPA] associated with higher total testosterone concentrations in men. |
| Meeker et al. (2010a) | Cross sectional | 167 | [uBPA] associated with estradiol:testosterone ratio (E2:T), follicle-stimulating hormone (FSH), and thyroid stimulating hormone. |
| Meeker and Ferguson (2011) | Cross sectional | 1346 | [uBPA] associated with altered thyroid hormones in adults and adolescents (NHANES 2007/08). |
| *Male reproduction* | | | |
| Li et al. (2009; 2010) | Cross sectional | 550; 427 | Occupational BPA exposure associated with male sexual dysfunction in both studies. |
| Li et al. (2011) | Cross sectional | 218 | Occupational BPA exposure associated with decreased sperm count. |
| Meeker et al. (2010b; 2011) | Cross-sectional | 190 | [uBPA] associated with decreased semen quality and DNA damage. |

| | | | |
|---|---|---|---|
| Mendiola et al. (2010) | Cross sectional | 302 | Non significant effect on reproductive function and sperm count. |
| *Endometriosis* | | | |
| Cobellis et al.(2009) | Case-Control | 69 | No association between [uBPA] and endometriosis. |
| *Breast Cancer* | | | |
| Yang et al. (2009) | Case-Control | 152 | [uBPA] were higher among cases compared with controls. |
| *Immune System* | | | |
| Clayton et al. (2011) | Cross sectional | 3728 | [uBPA] associated with higher cytomegalovirus antibody (NHANES 2003/06). |
| *CVD, diabetes, and obesity* | | | |
| Lang et al. (2008) | Cross sectional | 1455 | [uBPA] associated with CVD and diabetes (NHANES 2003-04). |
| Melzer et al. (2010) | Cross sectional | 1493 | [uBPA] associated with CVD but not with diabetes (NHANES 2005-06). |
| Melzer et al. (2012a) | Cross sectional | 591 | [uBPA] higher in those with severe coronary artery stenoses compared to those with no vessel disease. |
| Melzer et al. (2012b) | Case-Control | 1919 | [uBPA] associated with incident coronary artery disease (CAD). |
| Shankar and Teppala (2011) | Cross sectional | 3967 | [uBPA] associated with type-2 diabetes (NHANES 2003/08). |
| Silver et al. (2011) | Cross sectional | 4389 | [uBPA] association with type-2 diabetes driven only by data from 2003-04 NHANES cycle (NHANES 2003/08). |
| Carwile and Michels (2011) | Cross sectional | 2747 | [uBPA] associated with general obesity in adults (NHANES 2003/06). |
| Trasande et al. (2012) | Cross sectional | 2838 | [uBPA] associated with obesity in children and adolescents (NHANES 2003/08). |
| Wang et al. (2012) | Cross sectional | 3390 | [uBPA] associated with generalized obesity, abdominal obesity, and insulin resistance in adult Chinese population. |
| *Renal function* | | | |
| You et al. (2011) | Cross sectional | 2573 | [uBPA] decreased with decreasing renal function (NHANES 2003/06.) |

Figure 1.1 The InChianti study time-table. Urinary Bisphenol A was measured in samples collected at Baseline and nine years later (Follow-up 3).

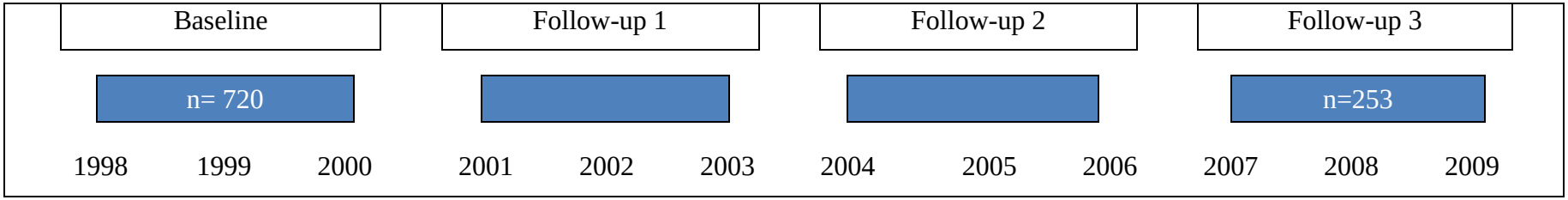
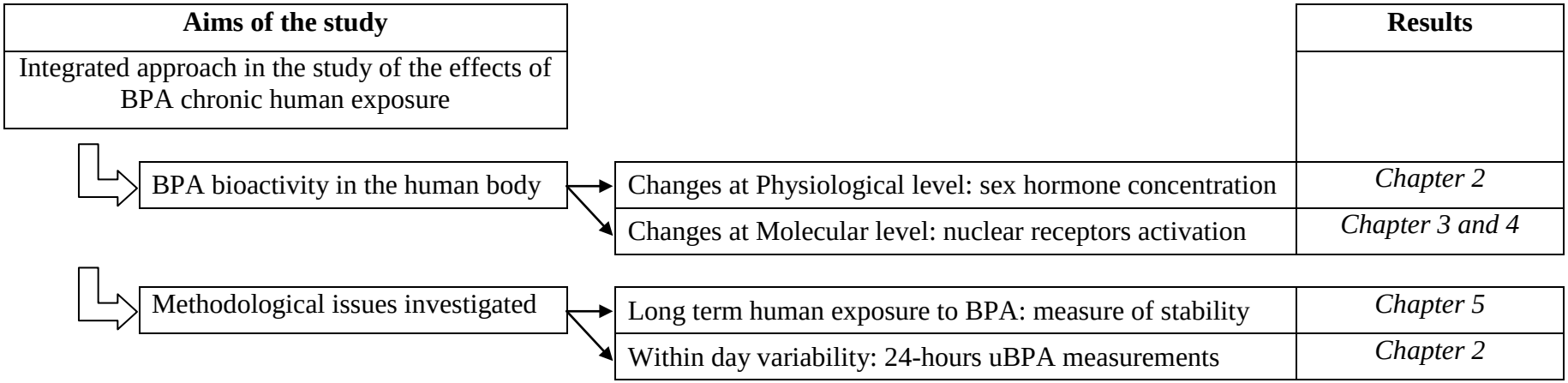


Figure 1.2 Schematic illustrating the aims of the research.



# 2. Daily Bisphenol A Excretion and Associations with Sex Hormone Concentrations: Results from the InChianti Adult Population Study.

*2.1 Statement of the candidate's contribution to the co-authored paper.*

The candidate had full access to all of the data in the study and was responsible for the statistical analysis: descriptive statistics, data tabulation, analyses of the association between uBPA concentration and parameters in the InChianti dataset were carried out during the first year of the research programme. Analysis and interpretation of the results were discussed with Prof. T. Galloway and Prof. D. Melzer who were responsible for the study design and the accuracy of the data analysis. The candidate also contributed to the drafting of the *Materials and Methods* and the *Results* sections as well as to the critical revision of the paper.

# Daily Bisphenol A Excretion and Associations with Sex Hormone Concentrations: Results from the InCHIANTI Adult Population Study

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**Background:** Bisphenol A (BPA) is a high production volume chemical widely used in packaging for food and beverages. Numerous studies have demonstrated that BPA can alter endocrine function in animals, yet human studies remain limited.

**Objective:** We estimated daily excretion of BPA among adults and examined hypothesized associations with serum estrogen and testosterone concentrations.

**Methods:** We conducted cross-sectional analyses using data from the InCHIANTI Study, a prospective population-based study of Italian adults. Our study included 715 adults between 20 and 74 years old. BPA concentrations were measured by liquid chromatography–mass spectrometry in 24-hr urine samples. The main outcome measures were serum concentrations of total testosterone and 17β-estradiol.

**Results:** Geometric mean urinary BPA concentration was 3.59 ng/mL [95% confidence interval (CI), 3.42–3.77 ng/mL], and mean excretion was 5.63 μg/day (5th population percentile, 2.1 μg/day; 95th percentile, 16.4 μg/day). We found higher excretion rates among men, younger respondents, and those with increasing waist circumference ($p = 0.013$) and weight ($p = 0.003$). Higher daily BPA excretion was associated with higher total testosterone concentrations in men, in models adjusted for age and study site ($p = 0.044$), and in models additionally adjusted for smoking, measures of obesity, and urinary creatinine concentrations (β = 0.046; 95% CI, 0.015–0.076; $p = 0.004$). We found no associations with the other serum measures. We also found no associations with the primary outcomes among women, but we did find an association between BPA and SHBG concentrations in the 60 premenopausal women.

**Conclusion:** Higher BPA exposure may be associated with endocrine changes in men. The mechanisms involved in the observed cross-sectional association with total testosterone concentrations need to be clarified.

**Key words:** endocrine disruption, androgen, antiandrogen, bisphenol A, human biomonitoring, health effects, InCHIANTI. *Environ Health Perspect* 118:1603–1608 (2010). doi:10.1289/ehp.1002367 [Online 25 August 2010]

Bisphenol A (BPA) is a synthetic compound that is a suspected endocrine disruptor—a compound capable of causing dysfunction to hormonally regulated body systems (Talsness et al. 2009). BPA is used as a monomer in polycarbonate plastics and in the epoxy resins that are used to line food and beverage containers; it is one of the world's highest production volume chemicals (Burridge 2003). Widespread and continuous daily exposure to BPA is believed to occur primarily through the diet (Stahlhut et al. 2009), as well as from drinking water, dental sealants, dermal exposure, and inhalation of household dusts. The presence of measurable concentrations of metabolites has been reported in the urine of > 90% of people in population-representative samples from across the globe (Calafat et al. 2008; Vandenberg 2007).

Most studies of the health effects of BPA have focused on its well-documented estrogenic activity, with reports of both estrogen agonist (Lee et al. 2003) and androgen antagonist activity (Bonefeld-Jørgensen et al. 2007; Lee et al. 2003; Okada et al. 2008). Suppression of aromatase activity has been observed in laboratory studies (Bonefeld-Jørgensen et al. 2007), as has binding to alternative nuclear receptors, including the aryl hydrocarbon receptor (Kruger et al. 2008) and estrogen-related receptor γ, the function of which remains unknown (Okada et al. 2008). In addition, BPA has been reported to cause thyroid hormone disruption (Moriyama et al. 2002), altered pancreatic beta-cell function (Ropero et al. 2008), and obesity-promoting effects (Newbold et al. 2008). The potential for low-dose effects has prompted debate on revising the current legislation of recommended safe daily exposure levels (Beronius et al. 2010; vom Saal et al. 2007).

Based on the animal and laboratory evidence, we previously hypothesized that higher urinary BPA concentrations would be associated with adverse human health effects. Using data from the U.S. National Health and Nutrition Examination Survey (NHANES) for 2003–2004, the first large-scale population-based epidemiological data on urinary BPA concentrations with sufficient power to detect low-dose effects, we showed for the first time a clear correlation between BPA exposure and disease in humans (Lang et al. 2008). Higher BPA concentrations in NHANES respondents were associated with diagnoses of cardiovascular disease and diabetes but not with other common diseases, which suggested specificity of the reported findings (Melzer et al. 2008, 2009). We recently used an entirely new study sample from the 2005–2006 NHANES to conduct an independent replication of the association of BPA and cardiovascular disease (Melzer et al. 2010). The results of this replication indicated that chance was an implausible explanation for our results.

Studies to clarify the mechanisms of these associations are clearly a priority. A substantive literature documents the disruption of circulating reproductive hormone concentrations after BPA exposures in animal models (reviewed by Richter et al. 2007; see also Bonefeld-Jørgensen et al. 2007; Goodman et al. 2009; Talsness et al. 2009). Studies of human populations have until now been limited to very small sample sizes. A significant, positive relationship was reported between circulating androgen concentrations and BPA exposure in a small study of 26 normal women and 47 women with ovarian dysfunction (Takeuchi et al. 2004). More recently,

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We thank the many people who contributed to the InCHIANTI study, including all of the anonymous participants.

R.C. was supported by University of Exeter internal funding. This project was supported in part by the (U.K.-government funded) Peninsula National Institute for Health Research (NIHR) Clinical Research Facility. It was also supported in part by the Intramural Research Program, National Institute on Aging, U.S. National Institutes of Health.

C.M. and P.M. are both employed by Brixham Environmental Laboratory, AstraZeneca UK Ltd., but their input was limited to conducting and documenting the bisphenol A (BPA) assays, and they were blind to the other data examined. The analysis of BPA samples on contract was funded from independent Peninsula College of Medicine and Dentistry sources. The remaining authors declare they have no actual or potential competing financial interests.

Received 29 April 2010; accepted 20 August 2010.

Meeker et al. (2010) studied serum thyroid and reproductive hormone levels in 167 men recruited through an infertility clinic and observed inverse relationships between urinary BPA concentrations and the free androgen index [ratio of testosterone to sex hormone–binding globulin (SHBG)], estradiol, and thyroid-stimulating hormone. Given these findings, we hypothesized that higher urinary BPA concentrations would be associated with altered reproductive hormone concentrations in serum. Because a limitation of previous studies has been their reliance on single spot urine samples, we based our current analysis on 24-hr urine collections, to provide a direct measure of daily excretion rates. We selected participants from the InCHIANTI study (Aging in the Chianti Area, Tuscany, Italy), a representive population-based study that was conducted in Chianti, Italy, from September 1998 to March 2000. Our analysis of the data from this sample provides the first report of daily BPA excretion levels in a large European cohort.

## Materials and Methods

***Study population.*** The InCHIANTI study (InCHIANTI 2010) was designed to identify risk factors for mid- and late-life morbidity and has been described extensively elsewhere (Ferrucci et al. 2000).

Briefly, InCHIANTI is a prospective population-based study of a suburban and rural town population. City registries were used to randomly select adults who were living in Greve in Chianti and in Bagno a Ripoli, Tuscany, Italy; a multistage sampling method was used (296 adults < 65 years old, 533 adults 65–74 years old, and 102 adults ≥ 75 years old; response rate, 91.6% from baseline interview). In line with previous work, we have limited our analysis here to participants ≤ 74 years old. The Instituto Nazionale Riposo e Cura Anziani Institutional Review Board provided ethical approval for the study. Participants gave informed consent, or if they were unable to do so, a close relative provided surrogate consent.

***Analysis of urinary BPA concentrations.*** Analysis of samples was performed (under contract) at the Brixham Environmental Laboratory, (Brixham, UK) in compliance with Good Laboratory Practice. Because orally administered BPA is considered to be rapidly and completely excreted, urine is the body fluid most appropriate for the biomonitoring assessment of BPA exposure (see Calafat et al. 2005). To measure total (free and conjugated) urinary concentrations of BPA, we used the methods employed by NHANES (Calafat et al. 2008) and adopted by the Division of Environmental Health Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention to prepare the urine samples; we performed the analyses using online solid-phase extraction (SPE) coupled with high-performance liquid chromatography (LC)–isotope dilution tandem mass spectrometry (MS/MS) with peak focusing. Analyses where carried out using a commercially available, integrated online SPE-LC system (Symbiosis Pharma System; Spark Holland BV, Emmen, the Netherlands) coupled with a triple-quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI) interface (TSQ Quantum Ultra AM; Thermo Scientific, Hemel Hempstead, UK). Two major advantages of the Symbiosis Pharma system are that a new SPE cartridge is used for every analysis and that one SPE cartridge is prepared while one is being analyzed. This system enabled a 7-min SPE-LC-HESI/MS/MS run time for each analysis point. A linear calibration was obtained from 0.50–100 µg/L ($R^2$ > 0.996). The limit of detection (LOD) was < 0.50 µg/L BPA, the limit of quantitation was 0.50 µg/L BPA, the lowest calibration standard with a signal height: noise height ratio > 10 (relative standard deviations < ±20%, all other standards < ±15%).

***Outcomes.*** Participants who consented to donate a blood sample were asked also to collect the urine for 24 hr in a vessel containing 3 g boric acid as preservative. During the 3 days before blood and urine collection, the subjects consumed a diet free of meat and fish. On the morning of the day before the blood samples were drawn, participants urinated and flushed away the first voided urine and then began the urine collection. During the day and night, all the produced urine was saved into the plastic bottle stored at room temperature or in the refrigerator. After 24 hr, bottles were weighed and the total volume measured in the clinic.

First thing the next morning, after having been sedentary for 15 min, fasting blood samples were collected for routine blood examination. Aliquots of serum and plasma were subsequently prepared and stored at –80°C for additional analyses. A 24-hr urine sample aliquot (70 mL) was stored at –20°C until further analyses.

Testosterone that circulates in the blood binds predominantly to protein, with approximately 40% bound to the high-affinity SHBG and 60% to albumin with lower affinity. Measurement of serum testosterone typically includes estimating total testosterone (free plus bound), free testosterone (not protein bound), and bioavailable testosterone (not SHBG bound).

Total testosterone was assessed through a commercial radioimmunological assay (RIA) kit (Active Testosterone RIA DSL-4000; Diagnostic Systems Laboratories, Webster, TX, USA, distributed by Chematil, s.r.l., Angri SA, Angri, Italy). The minimum LOD was 0.08 ng/mL. Intraassay coefficients of variation (CVs) for three different concentrations ranged from 7.8–9.6%, and interassay CVs ranged from 8.4–9.1%. Results were transformed and reported as nanograms per milliliter according to the manufacturer's instructions.

SHBG level was measured by RIA (IRMA DSL-7400; Diagnostic Products Corp., Los Angeles, CA, USA). The analytical sensitivity was 3 nmol/L. The intraassay CVs for three different concentrations were 1.1–3.7%, and interassay CVs were 8.7–11.5%.

Free testosterone was estimated from measured total testosterone, SHBG, and albumin (4.3 g/dL) using the method described by Vermeulen et al. (1999; for a worked example, see the International Society for the Study of the Aging Male 2010).

Estradiol levels were measured using an ultrasensitive RIA (Ultra-sensitive Estradiol RIA DSL-4800; Diagnostic Systems Laboratories, distributed by Chematil). The theoretical sensitivity was 2.2 pg/mL. Intraassay CVs across four different concentrations ranged from 6.5–8.9%, and interassay CVs ranged from 7.5–12.2% (at 108.7 pg/mL).

***Statistical analyses.*** Descriptive statistics of urinary BPA concentration and serum hormone levels were tabulated. We calculated geometric means and distribution percentiles of two different BPA measures. First, we measured the BPA volume concentration (expressed as micrograms of BPA/liter of urine). Then, we multiplied the BPA concentration by the urine collection rate (liters/day), which was measured considering the urine volume collected in 24 hr, and thus obtained the urinary excretion rate of BPA (expressed as micrograms/day).

We performed multivariate linear regression analyses to study the association between BPA and a broad range of demographic covariates and possible confounders. Because the concentrations of daily BPA excretion were not normally distributed, we used natural log transformation when BPA was considered the dependant variable. BPA values were not transformed when it was considered an explanatory variable in serum hormone examination. In all analyses, an upper age cutoff was 75 years to minimize the problem of comorbidity.

We adjusted our models by selecting different covariates. The variables included in our analyses were age, reported in years at the last birthday and used as a continuous variable; the two municipalities (study sites) where participants lived; waist circumference (centimeters) and weight (kilograms); and body mass index (BMI) was calculated as the weight (kilograms) divided by the square of height (meters). BMI was tested as a continuous variable and as a categorized dummy variable with subjects divided into underweight (< 18.5 kg/m$^2$), recommended weight (18.5–24.9 kg/m$^2$), overweight (25.0–29.9 kg/m$^2$), obese I (30.0–34.9 kg/m$^2$), and obese II (≥ 35 kg/m$^2$) categories. We also

considered smoking status, which appeared to be correlated to BPA in unadjusted models.

Urinary creatinine concentration is commonly used to adjust within-day variation in metabolite analysis from single spot urine samples (Barr et al. 2005). Linear regression analysis using the outcome (hormone) measures as the dependant variable was performed first considering all the subjects and then considering men and women separately. Data analysis was performed using STATA (version 10 SE; StataCorp LP, College Station, TX, USA); $p < 0.05$ was considered significant.

## Results

The geometric mean urinary concentration of BPA was 3.59 ng/mL [95% confidence interval (CI), 3.42–3.77 ng/mL; Table 1]. Based on the 24-hr urine collection, the daily excretion rate of BPA had a geometric mean of 5.63 μg/day but varied widely. The distribution was skewed, with a 10th percentile of 2.6 μg/day (95% CI, 2.5–2.8 μg/day) and a 90th percentile of 11.8 μg/day (95% CI, 10.9–12.7 μg/day). Daily BPA excretion was lower among women than amomg men ($p < 0.001$ in models adjusted for age, sex, and study site) and lower with advancing age ($p < 0.001$). We obtained identical results both with and without correction for creatinine. In models adjusted for age, sex, and study site (Table 2), we found no associations between daily BPA excretion and years of education or smoking status. We did find associations with waist circumference (β = 0.0062; 95% CI, 0.0016–0.0108; $p = 0.013$) and with weight (β = 0.0064; 95% CI, 0.0023–0.0104; $p = 0.003$).

In models for men, adjusted for age and study site, we found no association between BPA excretion and 17β-estradiol. However, we did find a significant association between daily BPA excretion and total testosterone concentration (β = 0.0237; 95% CI, 0.0006–0.0468; $p = 0.044$). In models adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine, the BPA association with total testosterone levels was highly significant (β = 0.046; 95% CI, 0.015–0.076; $p = 0.004$; Table 3).

To explore further the association with testosterone in men, we examined associations with the derived measure of free testosterone, based on SHBG concentrations. The association between BPA excretion and free testosterone narrowly missed significance ($p = 0.075$ in fully adjusted models).

For women, the geometric mean of the 17β-estradiol concentration was 6.89 pg/mL (Table 4), but this varied dramatically by menopause status: 22.4 pg/mL (95% CI, 16.7–30.0 pg/mL) in the 57 premenopausal women and 5.3 pg/mL (95% CI, 4.8–5.7 pg/mL) in the 290 postmenopausal women. In the models that tested hormone associations with BPA excretion among women (Table 4), we found no significant associations for either estradiol or total testosterone. Both SHBG concentration and the derived measure of free testosterone showed significant associations with BPA excretion in premenopausal women, although it should be noted that the method used (direct measure of free testosterone by RIA and calculation of the free androgen index) was not designed for measuring androgen

**Table 2.** Geometric means (GMs) of BPA excretion, by covariate status, plus age, sex, and study site using adjusted regression estimates of association.

| Variable | *n* (%) | GM (μg/day) (95% CI) | *p*-Value[a] |
|---|---|---|---|
| Education (years) | | | |
| 0 | 3 (0.4) | 4.72 (1.80 to 12.35) | (dropped) |
| 1–5 | 364 (50.9) | 5.06 (4.75 to 5.39) | —[b] |
| 6–8 | 152 (21.3) | 6.27 (5.59 to 7.02) | 0.071 |
| 9–13 | 123 (17.2) | 6.07 (5.46 to 6.75) | 0.968 |
| 14–19 | 63 (8.8) | 6.99 (5.99 to 8.17) | 0.170 |
| ≥ 20 | 10 (1.4) | 5.84 (3.82 to 8.94) | 0.576 |
| BMI category (kg/m$^2$) | | | |
| Underweight (BMI 0–18.5) | 4 (0.6) | 2.74 (1.29 to 5.81) | (dropped) |
| Normal (BMI 18.5–25) | 215 (30.1) | 5.67 (5.22 to 6.16) | —[b] |
| Overweight (BMI 25–30) | 314 (43.9) | 5.84 (5.43 to 6.27) | 0.296 |
| Obese I (BMI 30.1–34.9) | 138 (19.3) | 5.66 (5.04 to 6.34) | 0.369 |
| Obese II (BMI ≥ 35) | 32 (4.5) | 4.85 (3.94 to 5.98) | 0.738 |
| Unknown | 12 (1.6) | 3.46 (2.65 to 4.52) | (dropped) |
| Smoking history | | | |
| Never | 380 (53.2) | 3.20 (3.03 to 3.37) | —[b] |
| Former | 171 (23.9) | 3.76 (3.50 to 4.05) | 0.259 |
| Current | 164 (22.9) | 3.95 (3.62 to 4.32) | 0.773 |
| Continuous measures | | | |
| Waist circumference (cm) | 715 | β = 0.0062 (0.0016 to 0.0108) | 0.013 |
| Weight (kg) | 715 | β = 0.0064 (0.0023 to 0.0104) | 0.003 |
| Urinary creatinine concentration (mg/dL) | 715 | β = –0.0012 (–0.0025 to 0.0003) | 0.116 |

[a]Adjusted for age, sex, and site. [b]The base category against which the others are tested.

**Table 1.** Geometric mean (GM) and selected population percentiles of urinary BPA concentrations and daily excretion of the study sample.

| | | | Percentile (95% CI) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| BPA variable | *n* (%) | GM (95% CI) | 5th | 10th | 25th | 50th | 75th | 90th | 95th |
| All | | | | | | | | | |
| UER (μg/day) | 715 | 5.63 (5.37–5.90) | 2.1 (1.9–2.3) | 2.6 (2.5–2.8) | 3.7 (3.6–3.9) | 5.6 (5.1–5.8) | 8.3 (7.7–8.7) | 11.8 (10.9–12.7) | 16.4 (14.0–20.1) |
| Urinary concentration (ng/mL) | 720 | 3.59 (3.42–3.77) | 1.3 (1.2–1.4) | 1.6 (1.5–1.7) | 2.3 (2.1–2.4) | 3.5 (3.3–3.7) | 5.4 (5.0–5.9) | 8.0 (7.4–9.5) | 11.5 (10.3–13.7) |
| Sex | | | | | | | | | |
| Male | | | | | | | | | |
| UER (μg/day) | 332 (46.4) | 6.26 (5.87–6.68) | 2.5 (2.0–2.7) | 3.0 (2.6–3.3) | 4.3 (3.9–4.6) | 6.1 (5.7–6.8) | 9.0 (8.3–9.7) | 12.5 (11.7–15.4) | 16.7 (14.5–23.7) |
| Urinary concentration (ng/mL) | 334 (46.4) | 4.02 (3.76–4.31) | 1.5 (1.4–1.6) | 1.8 (1.6–2.0) | 2.4 (2.3–2.7) | 3.9 (3.6–4.3) | 6.3 (5.7–6.7) | 9.8 (8.1–10.9) | 13.0 (10.7–14.8) |
| Female | | | | | | | | | |
| UER (μg/day) | 383 (53.6) | 5.14 (4.81–5.49) | 2.0 (1.8–2.2) | 2.4 (2.2–2.6) | 3.5 (3.2–3.7) | 4.9 (4.5–5.3) | 7.3 (6.7–8.2) | 10.7 (9.9–12.3) | 14.4 (12.2–20.4) |
| Urinary concentration (ng/mL) | 386 (53.6) | 3.25 (3.04–3.47) | 1.1 (1.1–1.3) | 1.4 (1.3–1.6) | 2.1 (2.0–2.3) | 3.2 (2.9–3.4) | 4.7 (4.4–5.2) | 7.2 (6.5–7.8) | 11.0 (7.7–14.1) |
| Age group (years) | | | | | | | | | |
| 20–40 | | | | | | | | | |
| UER (μg/day) | 109 (15.2) | 6.61 (5.98–7.31) | 2.6 (2.3–3.2) | 3.2 (2.6–3.8) | 4.7 (4.0–5.3) | 6.7 (5.8–7.7) | 8.9 (8.3–10.9) | 12.5 (11.2–16.6) | 16.9 (12.6–24.1) |
| Urinary concentration (ng/mL) | 111 (15.4) | 4.31 (3.86–4.82) | 1.6 (1.2–2.1) | 2.1 (1.6–2.3) | 3.2 (2.4–3.6) | 4.4 (4.0–4.8) | 6.0 (5.6–6.8) | 8.3 (7.0–12.0) | 12.2 (8.4–17.4) |
| 41–65 | | | | | | | | | |
| UER (μg/day) | 157 (22.0) | 6.69 (6.04–7.40) | 2.7 (2.2–3.2) | 3.2 (2.8–3.6) | 4.6 (4.0–5.0) | 6.2 (5.6–6.9) | 9.2 (8.1–10.3) | 16.1 (11.3–21.3) | 23.8 (16.7–40.7) |
| Urinary concentration (ng/mL) | 157 (21.8) | 3.95 (3.53–4.42) | 1.4 (1.2–1.5) | 1.5 (1.4–2.0) | 2.4 (2.1–2.8) | 3.7 (3.3–4.4) | 5.8 (5.1–6.7) | 9.6 (7.5–15.3) | 16.7 (11.1–22.3) |
| 66–74 | | | | | | | | | |
| UER (μg/day) | 449 (62.8) | 5.10 (4.80–5.41) | 1.9 (1.6–2.1) | 2.4 (2.1–2.6) | 3.5 (3.1–3.6) | 4.9 (4.5–5.3) | 7.4 (7.0–8.3) | 10.9 (9.9–12.1) | 14.2 (12.2–17.1) |
| Urinary concentration (ng/mL) | 452 (62.8) | 3.32 (3.12–3.53) | 1.2 (1.1–1.3) | 1.5 (1.3–1.6) | 2.1 (2.0–2.3) | 3.2 (2.9–3.4) | 4.8 (4.4–5.6) | 7.6 (7.0–8.9) | 10.7 (9.3–12.8) |

concentrations in women, where the concentrations involved are at the very lowest LODs (Miller et al. 2004; Vermeulen et al. 1999).

***Sensitivity analysis.*** For a sensitivity analysis of our main finding, we examined the relationship between daily BPA excretion and total testosterone levels in men, excluding outlier BPA values above 25 μg/day ($n$ = 7 removed, ranging from 25.29–41.12 μg/day) (see Figure 1 for unadjusted model). In fully adjusted models as above, BPA excretion per day remained associated with total testosterone concentrations in men (β = 0.0521; 95% CI, 0.0172–0.08703; $p$ = 0.004).

Post hoc analyses for bioavailable testosterone showed patterns similar to those reported for free testosterone (data not shown). Associations with estradiol: testosterone ratios were nonsignificant.

## Discussion

In this study, we have reported for the first time the daily excretion levels of BPA among European adults in a large-scale and high-quality population-based sample. After adjusting for potential confounders, we have shown that higher BPA daily excretion was associated with an increase in serum total testosterone concentration in men.

These results are important because they provide the first report, using data from a large-scale human population, of associations between elevated exposure to BPA and alterations in circulating hormone levels. They also illustrate that the extent of exposure to BPA is similar in this European urban and rural population to exposures seen in the general adult population of the United States (Calafat et al. 2008). Previous studies of the relationship between human exposure to BPA and endocrine function are sparse and involve reported alterations in androgens (gonadotrophins or testosterone) in urine or serum in both men and women, although the numbers of participants were small (Hanaoka et al. 2002; Takeuchi and Tsutsumi 2002; Takeuchi et al. 2004). Hanaoka et al. (2002) studied 42 occupationally exposed male production workers and age-matched controls and showed that urinary BPA concentrations were inversely associated with follicle-stimulating hormone (FSH) but not with free testosterone or leutinizing hormone. In a later study of 167 men recruited through an infertility clinic (Thuillier et al. 2009), BPA concentrations in urine were positively associated with both FSH and FSH:inhibin ratio and inversely associated with estradiol:testosterone ratio. Because FSH and inhibin B are the two hormones considered most predictive of semen quality, Thuillier et al. (2009) concluded that BPA may have been associated with adverse effects on Sertoli cells or their FSH receptors that led to altered inhibin B production and reduced semen quality. In an animal study, rats exposed to BPA *in utero* did not show significant changes in circulating testosterone levels in adulthood, which suggests normal functioning of Leydig and Sertoli cells (Goodman et al. 2009). Because estrogens and androgens can exert differential effects in function depending on the cell type and its stage of development, the consequences of BPA exposure on adult reproductive and somatic tissues merits further attention.

Our results showed an association with total testosterone concentrations but no significant trend in 17β-estradiol levels with higher BPA excretion in men. The results reported by Meeker et al. (2010) are consistent with those reported here, although the positive trend ($p$ = 0.17) between BPA and testosterone reported by Meeker et al. (2010) did not reach statistical significance in their smaller study. Mendiola et al. (2010) reported finding no association between urinary BPA concentrations and testosterone levels in 375 male partners of pregnant women; in addition to differences in study group, their urinary BPA concentrations appear substantially lower than in our study sample.

Plausible explanations for our finding of an increase in total testosterone include a reduction in aromatase activity (Akingbemi et al. 2004; Huang and Leung 2009; Nativelle-Serpentini et al. 2003), which would lead to a decrease in the conversion of testosterone to estradiol. Because BPA has been shown to possess antiandrogenic activity (Bonefeld-Jørgensen et al. 2007; Lee et al. 2003), an alternative explanation could be that a blockade of androgen-binding sites alters feedback control mechanisms that leads to an increase in circulating testosterone. Lee et al. (2003) showed BPA to affect multiple steps in the activation and function of the androgen receptor, including noncompetitive inhibition of binding of endogenous androgens, nuclear localization, and transactivation, with uncertain consequences for androgen homeostasis. In our study, associations with the derived measure of free testosterone narrowly missed statistical significance.

Alternatively, there could be differential effects of BPA on the metabolism of testosterone and estrogen. A study of steroid hormone production in rat ovarian cells showed that BPA increased both testosterone synthesis and the mRNA expression of steroidogenic enzymes (Zhou et al. 2008). BPA also significantly decreased the activity of enzymes involved in the hydroxylation of testosterone, including the cytochrome P450 isoforms for testosterone 2 α-hydroxylase and testosterone 6 β-hydroxylase, CYP2C11/6 and CYP3A2/1, respectively, in isolated rat livers (Hanioka

**Table 3.** Simple and fully adjusted regression models of the associations between BPA (μg/day) and 17β-estradiol and testosterone concentrations for men.

| | | | Age and study-site adjusted | | Fully adjusted[a] | |
|---|---|---|---|---|---|---|
| Hormone | $n$ | Geometric mean (95% CI) | β-Coefficient (95% CI) | $p$-Value | β-Coefficient (95% CI) | $p$-Value |
| 17β-Estradiol (pg/mL) | 293 | 12.89 (12.26 to 13.56) | –0.00004 (–0.0086 to 0.0085) | 0.992 | 0.0002 (–0.011 to 0.011) | 0.975 |
| Total testosterone (ng/mL) | 307 | 4.55 (4.42 to 4.69) | 0.0237 (0.0006 to 0.0468) | 0.044 | 0.046 (0.015 to 0.076) | 0.004 |
| SHBG (nmol/mL) | 316 | 80.84 (76.60 to 85.30) | –0.0009 (–0.0095 to 0.0076) | 0.830 | 0.0011 (–0.0075 to 0.0096) | 0.805 |
| Free testosterone (ng/dL) | 316 | 4.72 (4.50 to 4.95) | 0.0081 (–0.0012 to 0.0175) | 0.089 | 0.0088 (–0.0009 to 0.0185) | 0.075 |

[a]Full models were adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration.

**Table 4.** Simple and fully adjusted regression models of the associations between BPA (μg/day) and 17β-estradiol, testosterone, and SHBG concentrations for women.

| | | | Age and study-site adjusted | | Fully adjusted[a] | |
|---|---|---|---|---|---|---|
| Hormone | $n$ | Geometric mean (95% CI) | β-Coefficient (95% CI) | $p$-Value | β-Coefficient (95% CI) | $p$-Value |
| Premenopause | | | | | | |
| 17β-Estradiol (pg/mL) | 57 | 22.4 (16.7–30.0) | –0.026 (–0.066 to 0.014) | 0.204 | –0.022 (–0.066 to 0.0229) | 0.325 |
| Total testosterone (ng/mL) | 61 | 0.69 (0.61–0.77) | –0.004 (–0.015 to 0.007) | 0.451 | –0.007 (–0.018 to 0.004) | 0.192 |
| SHBG (nmol/mL) | 60 | 134.3 (111.6–161.5) | 0.029 (0.004 to 0.054) | 0.024 | 0.038 (0.013 to 0.063) | 0.004 |
| Postmenopause | | | | | | |
| 17β-Estradiol (pg/mL) | 290 | 5.3 (4.8–5.7) | –0.002 (–0.010 to 0.005) | 0.516 | –0.003 (–0.010 to 0.005) | 0.448 |
| Total testosterone (ng/mL) | 294 | 0.54 (0.49–0.59) | –0.0003 (–0.0036 to 0.0030) | 0.871 | –0.001 (–0.004 to 0.002) | 0.555 |
| SHBG (nmol/mL) | 299 | 105.2 (98.8–112.1) | 0.002 (–0.004 to 0.008) | 0.541 | 0.003 (–0.003 to 0.009) | 0.272 |

[a]Full models adjusted for age, study site, smoking, BMI, weight, waist, and urinary creatinine concentration.

et al. 1998), both of which could lead to a net increase in circulating testosterone. The possibility that BPA could interfere with the RIA used to quantify serum testosterone is unlikely given the low cross-reactivity shown by the anti-testosterone antibody used in the assay and is further discounted by mathematical modeling studies showing negligible effects of xenoestrogens on the displacement of bound hormone and tracer during binding and extraction steps *in vitro* (Heringa et al. 2004).

It is also plausible that an androgenic environment leads to alterations in the metabolism of BPA, that is, reverse causation. Metabolism of BPA in the intestine and liver catalyzed by uridine diphosphate-glucuronosyl transferase (UGT) yields the major urinary metabolite BPA-glucuronide (Teeguarden et al. 2005). The level of both UGT activity and transcription has been shown to be downregulated by androgens (Guillemette et al. 1997; Takeuchi et al. 2004), which could result in an increase in serum BPA concentration under hyperandrogenic conditions. However, it is unlikely that such metabolic change could alter 24-hr urinary BPA excretion in the context of repeated ingestion of BPA at the population level and the limited increase in testosterone concentrations evident in our analysis.

Urinary BPA concentrations have previously been reported in 100 pregnant European women, with 82% of the study population showing detectable levels of BPA, median concentration 1.2 ng/mL (Ye et al. 2008). This concentration is lower than the mean value presented here, 3.59 ng/mL (95% CI, 3.42–3.77 ng/mL), although there are differences in age and sex profiles. Most studies have reported values from spot urine samples with or without correction for creatinine, with mean concentrations around 3 ng/mL (Dekant and Volkel 2008; Vandenberg et al. 2010) and 95th percentiles in the range of 11.5 ng/mL (Ye et al. 2008) to 16 ng/mL (Calafat et al. 2005). Here, we used 24-hr urine collection to calculate a mean daily excretion rate of 5.63 µg/day (95% CI, 5.67–5.90 µg/day).

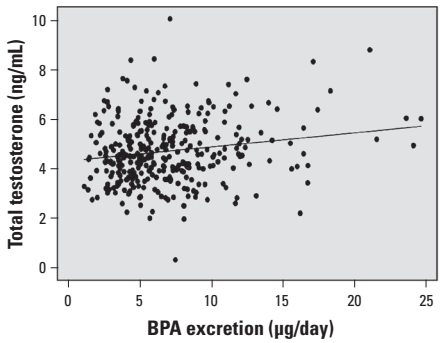


**Figure 1.** Scatter plot of BPA excretion per day against total testosterone concentrations, with unadjusted linear regression line (BPA outlier values censored at < 25 µg/day).

In an earlier Japanese study, Arakawa et al. (2004) reported median daily urinary excretions of BPA of 1.3–5.0 µg/day, with a maximum daily intake of BPA per body weight of 0.23 µg/kg/day based on 24-hr urine samples collected from 36 men; the median daily uptake was given as 0.02 µg/kg body weight. In controlled, acute human exposure studies, peak urinary concentrations of BPA metabolites were 4,500–6,800 µg/L 6 hr after oral administration of 60–80 µg/kg body weight. Based on these figures and assuming complete and rapid excretion, Dekant and Volkel (2008) suggested that a daily excretion rate of around 5 µg/L, as seen in the general population, indicates ingestion of < 25 µg of BPA in the hours prior to sampling (the maximum daily reference dose is 50 µg/kg/day). However, there are no actual *in vivo* data on the rate at which unconjugated BPA is converted to BPA-glucuronide in humans, only estimates. BPA is lipophilic with a log octanol–water partition coefficient (log $K_{ow}$) between 2.2 and 3.82, and it may partition to lipid-rich tissues, a suggestion supported by population-based half-lives for BPA calculated by Stahlhut et al. (2009) to be significantly longer than previous predictions of 6 hr. Given the correlations with BMI and waist circumference seen here, a true estimation of exposure rates remains a priority.

There are limitations to this study that should be borne in mind when interpreting the results. First, replication is required in an independent study population to exclude chance as an explanation, although the small *p*-value in fully adjusted models and the broad consistency with previous work suggest this is unlikely. Second, the analysis is based on a single day of BPA excretion, which is clearly not a perfect measure of longer term exposure given that human health effects are most likely associated with long-term low-dose exposure. However, using the 24-hr urine specimens is likely to be more accurate than previously published work, which has been based on spot urine samples with post hoc adjustment to try to correct for concentration effects. Spot urine samples themselves have been shown to be moderately sensitivity for predicting an individual's tertile categorization (Mahalingaiah et al. 2008). Misclassification due to this single-day snapshot of excretion will have resulted in a smaller (diluted) estimate of the strength of association between BPA and total testosterone concentrations: the true associations are likely to be much stronger.

Third, the cross-sectional nature of the association reported here needs to be treated with caution. It is also theoretically possible, for example, that those with higher testosterone concentrations alter their diet in such a way as to increase BPA exposure, or, as noted above, that higher testosterone concentrations are themselves responsible for altering metabolism of BPA. It is unclear, however, why altered metabolism would alter our measure of 24-hr excretion systematically, because all BPA is thought to be excreted in the urine in humans sooner or later. We previously reported positive associations between urinary BPA and prevalence of cardiovascular disease (Lang et al. 2008; Melzer et al. 2010). The relationship between circulating testosterone and cardiovascular risk remains to be comprehensively established, although an increased risk of cardiovascular adverse events was recently reportedly in a trial of testosterone supplementation in older men (Basaria et al. 2010).

Future work needs to replicate the association found and to clarify the mechanisms involved. Showing that raised BPA levels precede the increase in testosterone concentrations would establish the temporal sequence of changes and exclude reverse causation. However, a concurrent change in testosterone levels with BPA exposure would remain biologically important. A large-scale exposure trial may be necessary to clarify the association we identified, although the logistics and ethics of such a trial would require careful thought.

## Conclusions

Mean daily exposure to BPA among an Italian adult population sample is in line with previous estimates from the United States, with wide variations around the mean. We found an association between higher daily excretion of BPA and total testosterone concentrations among men. The mechanisms involved in this possible endocrine disruption need clarification.

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# 3. Bisphenol A Exposure Is Associated with in Vivo Estrogenic Gene Expression in Adults.

*3.1 Statement of the candidate's contribution to the co-authored paper.*

The candidate was responsible for laboratory and data analysis: qPCR gene expression analysis on the target genes, data normalization and tabulation, epidemiological analyses of the association between uBPA concentration and gene expression data were all carried out during the first and second year of the research programme. Analysis and interpretation of the results were discussed with Prof. T. Galloway, Dr. L. Harries, and Prof. D. Melzer who were responsible for the study design and the accuracy of the data analysis. The candidate also contributed significantly to the drafting of the manuscript and its critical revision.

# Bisphenol A Exposure Is Associated with *in Vivo* Estrogenic Gene Expression in Adults

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**Background:** Bisphenol A (BPA) is a synthetic estrogen commonly used in polycarbonate plastic and resin-lined food and beverage containers. Exposure of animal and cell models to doses of BPA below the recommended tolerable daily intake (TDI) of 50 µg/kg/day have been shown to alter specific estrogen-responsive gene expression, but this has not previously been shown in humans.

**Objective:** We investigated associations between BPA exposure and *in vivo* estrogenic gene expression in humans.

**Methods:** We studied 96 adult men from the InCHIANTI population study and examined *in vivo* expression of six estrogen receptor, estrogen-related receptor, and androgen receptor genes in peripheral blood leukocytes.

**Results:** The geometric mean urinary BPA concentration was 3.65 ng/mL [95% confidence interval (CI): 3.13, 4.28], giving an estimated mean excretion of 5.84 µg/day (95% CI: 5.00, 6.85), significantly below the current TDI. In age-adjusted models, there were positive associations between higher BPA concentrations and higher *ESR2* [estrogen receptor 2 (ER beta)] expression (unstandardized linear regression coefficient = 0.1804; 95% CI: 0.0388, 0.3221; $p = 0.013$) and *ESRRA* (estrogen related receptor alpha) expression (coefficient = 0.1718; 95% CI: 0.0213, 0.3223; $p = 0.026$): These associations were little changed after adjusting for potential confounders, including obesity, serum lipid concentrations, and white cell subtype percentages. Upper-tertile BPA excretors (urinary BPA > 4.6 ng/mL) had 65% higher mean *ESR2* expression than did lower-tertile BPA excretors (0–2.4 ng/mL).

**Conclusions:** Because activation of nuclear-receptor–mediated pathways by BPA is consistently found in laboratory studies, such activation in humans provides evidence that BPA is likely to function as a xenoestrogen in this sample of adults.

**Key words:** bisphenol A, endocrine disruption, estrogen receptor-β, estrogen-related receptor-α, human biomonitoring, InCHIANTI, toxicogenomics. *Environ Health Perspect* 119:1788–1793 (2011). http://dx.doi.org/10.1289/ehp.1103809 [Online 10 August 2011]

Bisphenol A (BPA) is a synthetic compound that is suspected to act as an endocrine disruptor (i.e., a compound capable of causing dysfunction to hormonally regulated body systems) (Talsness et al. 2009). It was originally synthesized as a synthetic estrogen (Dodds and Lawson 1936). It is used extensively as a monomer in polycarbonate plastics and in the epoxy resins that are used to line food and beverage containers and is one of the world's highest-production-volume chemicals (Ritter 2011). Ubiquitous exposure to BPA is believed to occur mainly through the diet, with additional contributions from dental sealants, dermal exposure, and inhalation of household dusts. BPA metabolites have been reported in the urine of > 90% of people in representative population samples in the United States and Europe (Calafat et al. 2008; Galloway et al. 2010).

Whether BPA can cause human health effects is a matter of some debate. There has been concern about the potential for a relationship between BPA and negative health effects, including increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit–hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, and an increase in hormonally mediated cancers, such as prostate and breast cancers (reviewed by Hengstler et al. 2011; vom Saal et al. 2007). Cross-sectional epidemiological studies have shown higher BPA exposure to be associated with adverse health effects in the general adult population. In a study of 1,455 respondents in the 2003–2004 U.S. population-representative National Health and Nutrition Examination Survey (NHANES), higher urinary BPA concentrations were associated with cardiovascular disease diagnoses and with diagnosed diabetes but not with other common diseases, suggesting specificity of the reported findings (Lang et al. 2008). In a further study of data from NHANES 2005/2006, higher BPA concentrations were again associated with coronary heart disease, providing independent replication of the findings (Melzer et al. 2010). Higher exposure to BPA has also been associated with reproductive and developmental abnormalities. In a study of 249 mothers and their children, prenatal urinary BPA concentrations in mothers were prospectively associated with externalizing behavior scores among their children when measured at 2 years of age (Braun et al. 2009). A positive association was also shown between BPA exposure and recurrent miscarriage in a prospective study of 67 women (Sugiura-Ogasawara et al. 2005). The mechanisms underlying these potential health effects remain to be determined.

Most studies of the health effects of BPA have focused on its estrogenic activity because it is widely documented to function as an agonist of certain estrogen receptors (ERs) (Lee et al. 2003) and as an androgen antagonist and to suppress aromatase activity (Bonefeld-Jørgensen et al. 2007). Additional receptor-mediated biological activities, including binding to the orphan estrogen-related receptor ERRγ (Okada et al. 2008), thyroid hormone disruption (Moriyama et al. 2002), altered pancreatic β-cell function (Ropero et al. 2008), and obesity-promoting effects (Newbold et al. 2008), have been reported in different model systems. Many of these effects are already detectable in the nanomolar range, prompting calls for a revision to the current tolerable daily intake (TDI) of 50 µg/kg/day. However, until now, there has

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We thank all who contributed to the InCHIANTI study, including the anonymous participants.

R.C. was supported by University of Exeter internal funding. This project was supported in part by the (U.K. government–funded) Peninsula NIHR (National Institute for Health Research) Clinical Research Facility; the Intramural Research Program, of the National Institute on Aging, U.S. National Institutes of Health; and the European Centre for Environment and Human Health, University of Exeter.

C.M., P.M., and A.Y. are employed by Brixham Environmental Laboratory, AstraZeneca UK Ltd. Their input was limited to conducting and documenting the bisphenol A (BPA) assays, and they were blind to the other data examined. The analysis of BPA samples on contract was funded from independent Peninsula College of Medicine and Dentistry sources. The authors declare that they have no other actual or potential competing financial interests.

Received 12 April 2011; accepted 10 August 2011.

been no evidence that BPA at these low levels exerts significant biological effects in humans, and hence the TDI has remained unaltered (European Food Safety Authority 2010).

A recent cross-sectional examination of circulating sex hormone concentrations in 307 men showed higher BPA levels to be associated with changes in total testosterone concentrations [β = 0.046; 95% confidence interval (CI): 0.015, 0.076; $p = 0.004$ in fully adjusted models] (Galloway et al. 2010). There was no significant trend in 17β-estradiol levels with higher BPA in men, although an earlier study of 167 men recruited through an infertility clinic used multiple adjusted regression models to show BPA concentrations in urine to be inversely associated with the estradiol:testosterone ratio (Meeker et al. 2010). Plausible explanations for these endocrine changes include altered expression of hormone-responsive genes. To date there is no *in vivo* evidence for changes in sex-hormone–responsive gene expression associated with human exposure to BPA.

Here, we aimed to test the hypothesis that exposure to BPA would be associated with changes in the *in vivo* expression of estrogen- and androgen-responsive genes. To do this, we conducted a cross-sectional study to characterize six candidate estrogen- or androgen-related transcripts for differential *in vivo* expression in response to BPA exposure. The study population was selected from the InCHIANTI study, a large European population representative sample based in Chianti, Italy.

## Materials and Methods

***Study population.*** The InCHIANTI study, a prospective population-based study of Italian adults (InCHIANTI 2011), was designed to identify risk factors for mid- and late-life morbidity in urban and rural populations and has been described extensively elsewhere (Ferrucci et al. 2000). InCHIANTI is performed in two sites: Greve in Chianti (11,709 inhabitants) and Bagno a Ripoli (Village of Antella, 4,704 inhabitants). The final study population included 1,453 persons (age range 20–102 years) stratified across age ranges using a multistage sampling process, with a response rate of 91.6% from the baseline interview. Subjects and specimens selected for the present study were those with the most adequate RNA and urine specimens in the 2008/2009 follow-up, and ≤ 76 years of age, in line with previous work. Women were excluded from this analysis because of cyclic hormonal variations in premenopausal subjects. The Instituto Nazionale Riposo e Cura Anziani Institutional Review Board (Florence, Italy) provided ethical approval. All participants gave informed (or surrogate) consent.

***Sample collection.*** Participants who consented to give a blood sample were also asked to provide a spot morning urine sample, which was stored at –20°C until further analysis. First thing in the morning on the day of the study visit, after participants had been sedentary for 15 min, fasting blood samples were collected for routine blood examination, and peripheral blood specimens preserving *in vivo* RNA expression were collected using PAXgene technology (Debey-Pascher et al. 2009).

***Analysis of urinary BPA concentrations.*** Samples were analyzed at the Brixham Environmental Laboratory Division of Analytical Chemistry (a division of AstraZeneca PLC; Brixham, UK) in compliance with Good Laboratory Practice, EU Directive 88/32/EEC (United Kingdom 2004). BPA ingested in humans is almost completely metabolized and rapidly excreted, so urine is considered the most appropriate matrix for assessment of exposure (Calafat et al. 2005). As part of our extensive Good Laboratory Practice–compliant quality control, we included reagent blanks and confirmed that samples stored for up to 10 years contained predominantly metabolized compound, confirming minimal leaching of BPA from collection or storage vessels during this time. BPA concentrations were measured in spot urine samples by liquid chromatography–mass spectrometry. Total (free and conjugated) urinary concentrations of BPA were obtained using online, solid-phase extraction coupled with high-performance liquid chromatography–isotope dilution tandem mass spectrometry with peak focusing, as described previously (Galloway et al. 2010). Calibration was linear from 0.50 to 100 μg/L ($R^2 > 0.996$), limit of detection was < 0.50 ng/mL BPA, the limit of quantification was 0.50 ng/mL BPA, and the lowest calibration standard gave a signal height:noise ratio > 10 (relative standard deviations < 20%, all other standards < 15%).

***Gene expression by real-time reverse-transcriptase polymerase chain reaction (RT-PCR).*** Blood leukocytes were used for transcript analysis because they are convenient and available and because they provide sufficient power in large cohorts where access to other tissues is lacking. Because BPA is metabolized in the intestines and liver to form predominantly BPA-monoglucuronide, which is passed through the bloodstream to the kidney, exposure of leukocytes to BPA and/or its metabolites is inevitable. To test the hypothesis that exposure to BPA would be associated with changes in the expression of estrogen- and androgen-responsive genes, we correlated BPA levels as a continuous trait with the expression of ER, androgen receptor (AR), and estrogen-related receptor (ERR) genes by quantitative real-time PCR in a subset of 100 male subjects. These genes were chosen because the nuclear hormone receptors they encode are transcription factors that control essential developmental and physiological pathways and because activation of these nuclear-receptor–mediated pathways by BPA is consistently found in laboratory studies.

Total RNA (100 ng) was reversed transcribed in 20 μL reactions using the Superscript III VILO kit (Invitrogen, Paisley, UK), according to the manufacturer's instructions.

The expression levels of *ESR1* (estrogen receptor 1; ERα), *ESR2* [estrogen receptor 2 (ER beta); ERβ], *ESRRA* (estrogen related receptor alpha; ERRα), *ESRRB* (estrogen related receptor beta; ERRβ), *ESRRG* (estrogen related receptor gamma), and *AR* (androgen receptor) genes were then assessed relative to the endogenous control genes *GUSB* (glucuronidase, beta) and *ACTB* (actin, beta; β-actin) on the TaqMan Low Density Array (TLDA) platform (Applied Biosystems, Foster City, CA, USA). Probes were inventoried with Applied Biosystems assays Hs01046812_m1, Hs01100358_m1, Hs01584024_m1, Hs00155006_m1, Hs00907244_m1, Hs99999908_m1, and Hs03023943_g1 for *ESR1*, *ESR2*, *ESRRB*, *ESRRG*, *AR*, *GUSB*, and *ACTB* genes, respectively. These probes were chosen because they are documented to pick up all isoforms and splice variants for the genes of interest.

The expression of the *ESRRA* gene was assessed by the use of a custom assay (probe and primer sequences available on request). Reaction mixes included 50 μL 2× TaqMan universal master mix (no AMPerase; Applied Biosystems), 40 μL distilled $H_2O$, and 10 μL cDNA template per TLDA loading port. PCR amplifications were performed on the ABI 7900HT platform (Applied Biosystems). Cycling conditions were 50°C for 2 min, 94.5°C for 10 min followed by 40 cycles of 97°C for 30 sec and 57.9°C for 1 min. The expression of each gene was measured in triplicate for each sample. Gene expression relative changes were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) relative to the geometric mean of the endogenous controls listed above using the StatMiner relative quantification software for high-throughput integrated analysis of TLDA data (Integromics, Grenada, Spain).

***Statistical analysis.*** We assessed the association of candidate gene expression levels with urinary BPA concentration by multivariable linear regression. Data were adjusted for potential confounding factors that could influence BPA exposure or candidate gene expression: age (reported in years at the last birthday and used as a continuous variable); body mass index (BMI) calculated as weight in kilograms divided by height in meters squared; waist circumference (as a continuous trait); highest level of education attained (in four categories: none/elementary, secondary, high school, and university/professional); low-density lipoprotein (LDL) cholesterol

(milligrams per deciliter); triglycerides (milligrams per deciliter); and study site [individuals were drawn from a rural village (Greve) and an urban population (Bagno a Ripoli)]. Models were also adjusted for the percentage of neutrophils (neutrophil%), lymphocytes (lymphocyte%), monocytes (monocyte%), and eosinophils (eosinophil%) [the percentage of basophils (basophil%) was not added because the cell subtype percentages would have equaled 100%].

The expression value of each of the target genes was not normally distributed, and we used natural log transformation when gene expression was considered as a dependent variable. In all analyses, an upper age cutoff was 76 years to minimize the problem of comorbidity. Data analysis was performed using STATA (version 10 SE; StataCorp LP, College Station, TX, USA); $p < 0.05$ was considered significant.

We used generalized additive models with penalized cubic regression splines (Wood 2006) to explore the functional form of the relationship between candidate gene expression levels and urinary BPA concentration. Linearity of the relationship between log-transformed expression level and log-transformed BPA concentration was assessed by visual inspection of the estimated spline functions and by examining the estimated degrees of freedom (edf) for the smoothed BPA term. Values of the edf close to 1 were taken as evidence of linearity. Adjustment was made for the same potential confounding factors that were included in the multivariable linear regression models. The prediction error criterion for smoothness selection was generalized cross-validation. Robustness of the smoothness selection was assessed by making comparisons with the use of maximum likelihood estimation. The spline models were fitted using R statistical software using the mgcv package for generalized additive modeling (version 2.12.1; R Project for Statistical Computing 2010).

**Table 1.** Characteristics of the sample ($n = 96$).

| Characteristic | Mean ± SD (range)[a] |
|---|---|
| Age (years) | 58.3 ± 15.2 (32–76) |
| Site (%) | |
| Greve | 38.4 |
| Bagno a Ripoli | 61.5 |
| Education (%) | |
| None/elementary | 22.9 |
| Secondary school | 26.0 |
| High school | 35.4 |
| Professional/university | 16.6 |
| BMI (kg/m$^2$) | 27.8 ± 4.1 (18.38–42.99) |
| LDL cholesterol (mg/dL) | 125.4 ± 29.8 (60–220) |
| Triglycerides (mg/dL) | 137.3 ± 75.3 (45–469) |
| Neutrophils% | 55.2 ± 9.7 (26.2–79.1) |
| Lymphocytes% | 32.8 ± 9.2 (9.1–59.9) |
| Monocytes% | 8.4 ± 2 (4.3–21.3) |
| Eosinophils% | 3 ± 1.7 (0.1–10.3) |
| Basophils% | 0.5 ± 0.2 (0.1–1.4) |

[a]Values shown are mean ± SD (range) except where indicated.

## Results

The sample ($n = 96$; Table 1) had a mean age of 58.3 years (range, 32–76 years) and a mean (± SD) BMI of 27.8 ± 4.1 kg/m$^2$. The geometric mean urinary BPA concentration was 3.65 ng/mL (95% CI: 3.13, 4.28) ranging from 0.73 to 56.94 ng/mL (limit of detection < 0.5 ng/mL). The distribution was skewed, with a 10th percentile of 1.3 ng/mL and a 90th percentile of 10.4 ng/mL. The estimated mean excretion was 5.84 µg/day (95% CI: 5.00, 6.85).

The expression of transcripts associated with sex-hormone–related signaling was quantified by real-time RT-PCR (Table 2). Expression of *ESRRG* was not detected in our samples. There was only one significant correlation of expression intensities between probes: between *ESR1* and *ESR2* (pairwise correlation = 0.24; $p = 0.02$). We obtained valid expression intensity measures for 96 men for the *ESR2* gene and 83 men for the *ESRRA* gene (Table 2). BPA concentrations in the 96 respondents with successful *ESR1* expression measures were no different from the remaining 55 respondents < 76 years of age (age-adjusted regression with log-transformed BPA concentration: unstandardized linear regression coefficient = 0.012; 95% CI: –0.114, 0.138; $p = 0.848$) for which measured BPA values were available.

Using urinary BPA concentrations as a continuous variable, we tested linear associations between BPA and gene expression. In age-adjusted regression models of log-transformed BPA concentrations against log-transformed expression levels (Table 3), we observed positive associations with *ESR2* (ERβ; coefficient = 0.1804; 95% CI: 0.0388, 0.3221; $p = 0.013$) and *ESRRA* (ERRα, coefficient = 0.1718, 95% CI: 0.0213, 0.3223, $p = 0.026$) but not with *ESR1*(ERα), *ESRRB*(ERRβ), or *AR*.

In models additionally adjusted for previously suggested confounders (Sharpe 2010) (BMI, LDL cholesterol and triglyceride concentrations, study site, and educational attainment—a proxy for social position) and white cell subtype percentages, the results were little changed: for *ESR2*, coefficient = 0.1387; 95% CI: 0.001, 0.2764; $p = 0.048$; for *ESRRA* coefficient = 0.1886; 95% CI: 0.0324, 0.3448; $p = 0.019$) (Table 4).

When using an alternative exposure metric of dividing BPA concentrations into tertiles in the fully adjusted models (Figure 1), participants in the lowest BPA exposure tertile had a geometric mean expression of *ESR2* of 0.80 IU (95% CI: 0.65, 0.99), rising to 1.32 IU (95% CI: 1.08, 1.60) in the highest tertile, a 65% increase in mean expression. For *ESRRA*, the same measures were 0.66 IU (95% CI: 0.49, 0.89) and 0.91 IU (95% CI: 0.78, 1.06), a 38% increase in mean expression of the gene.

Figure 2A shows a spline plot for the change in natural log of *ESR2* expression as a function of log-transformed urinary BPA concentration. This suggests that the positive association between *ESR2* and BPA concentration is curvilinear (edf = 1.45; *p*-value for smoothed term = 0.027), with evidence of a diminishing effect as BPA concentration increases. A similar spline plot for *ESRRA* expression is shown in Figure 2B. This suggests that the relationship with BPA concentration is linear for this ERR (edf = 1.00; *p*-value for smoothed term = 0.017).

**Table 2.** Expression characteristics of the tested estrogen and androgen target genes.

| Target gene | Assay ID[a] | Accession number[b] | *n* | Mean ± SD (range) |
|---|---|---|---|---|
| *ESR1* | Hs01046812_m1 | NM_000125 | 96 | 1.21 ± 0.535 (0.365–3.165) |
| *ESR2* | Hs01100358_m1 | NM_001040275 | 96 | 1.294 ± 0.899 (0.167–5.585) |
| *ESRRA* | Hs01067166_g1 | NM_004451 | 83 | 0.882 ± 0.33 (0.105–1.991) |
| *ESRRB* | Hs01584024_m1 | NM_004452 | 96 | 2.974 ± 2.434 (0.000–10.363) |
| *ESRRG* | Hs00155006_m1 | NM_206595 | | Not expressed |
| *AR* | Hs00907244_m1 | NM_000044.2 | 96 | 1.232 ± 0.673 (0.188–3.295) |

[a]TaqMan Gene Expression assay identification number. [b]Accession numbers from the National Center for Biotechnology Information (2011).

**Table 3.** Estimates for the associations between natural log of urinary BPA concentrations and gene expression intensity (log transformed), in age-adjusted and fully adjusted[a] regression models.

| | Age-adjusted model | | | Fully adjusted model | | |
|---|---|---|---|---|---|---|
| Gene | Coefficient (95% CI) | *p*-Value | Std β | Coefficient (95% CI) | *p*-Value | Std β |
| *ESR1* | –0.0657 (–0.1815, 0.0500) | 0.262 | –0.117 | –0.1071 (–0.2205, 0.0063) | 0.064 | –0.1909 |
| *ESR2* | 0.1804 (0.0388, 0.3221) | 0.013 | 0.231 | 0.1387 (0.001, 0.2764) | 0.048 | 0.1775 |
| *ESRRA* | 0.1718 (0.0213, 0.3223) | 0.026 | 0.250 | 0.1886 (0.0324, 0.3448) | 0.019 | 0.2699 |
| *ESRRB* | –0.2816 (–1.3969, 0.8337) | 0.617 | –0.054 | –0.4857 (–1.6669, 0.6955) | 0.416 | –0.0925 |
| *ESRRG* | ND | | | ND | | |
| *AR* | 0.0115 (–0.1404, 0.1634) | 0.881 | 0.016 | 0.0925 (–0.0646, 0.2495) | 0.245 | 0.1285 |

Abbreviations: ND, not detected; Std, standardized.
[a]Full adjustment included age, BMI, study site, educational attainment, and LDL cholesterol and triglyceride concentrations, plus percentages of neutrophils, lymphocytes, monocytes, and eosinophils.

## Discussion

In this study, we aimed to assess whether increased urinary BPA concentrations were associated with changes in gene expression *in vivo* in the general adult population. We made use of a large-scale and high-quality population-representative data set for which specimens preserving *in vivo* RNA expression were available. We were able to measure *in vivo* expression of five ER, ERR, and AR genes in peripheral blood leukocytes in 96 adult men. Using urinary BPA excretion as a marker of exposure, we found that those with higher BPA exposures had higher expression of two estrogen-responsive genes, *ESR2* (ERβ) and *ESRRA* (ERRα).

These findings are important because they suggest that BPA is bioactive in the human body and that associations with hormone signaling and related disorders are biologically plausible. ERβ, which showed the strongest association with BPA exposure, is one of two ER subtypes that, along with ERα, mediates the physiological actions of estrogens (Swedenborg et al. 2009). ERβ and ERα have distinct tissue distribution, ligand specificities, and functions; ERα is predominant in the regulation of female reproduction, whereas ERβ is important in maintaining the structure and function of nonclassic target tissues, including prostate, colon, and cardiovascular and central nervous systems (Imamov et al. 2005). BPA displays estrogenic agonist activities against both ERα and ERβ subtypes *in vitro*, with relatively high ERβ selectivity (Matthews et al. 2001), consistent with our findings. The modulation by BPA of ER gene expression has previously been shown in animal models, at environmentally relevant concentrations. For example, exposure of rat prostate mesenchyme cells to 1 nM BPA led to altered ER gene expression, accompanied by modest stimulation of cell growth, with a threshold of induction around 30-fold less potent than 17β-estradiol (Richter et al. 2007).

ERRα belongs to the NR3B orphan nuclear receptor subgroup, which consists of ERRα, ERRβ, and ERRγ (Hong et al. 1999). All three ERRs show close sequence identity to the ERα DNA binding domain and also feature a conserved C-terminal domain with a putative ligand binding domain and interaction surfaces for coregulators, and a less conserved N-terminal domain (Giguère 2002). Despite this close structural homology to the ERs, estradiol does not bind to ERRα, and X-ray crystallography of the putative ligand-binding domain pocket of ERRα shows it to be almost completely occupied by side chains. This supports the suggestion that ERRα shows ligand-independent transcriptional activation and is largely dependent on its functional interaction with coregulators, including peroxisome proliferator-activated receptor-γ (PPARγ) coactivator 1α (PGC-1A) and PGC-1B for optimal gene regulation (Ranhotra 2010). In adults, ERRα is constitutively expressed in tissues that preferentially use fatty acids as energy sources, including adipose tissue, heart, and skeletal muscle, where it plays a significant role in regulating energy homeostasis and adaptive oxidative capacity (Dufour et al. 2007). These functions are thought to involve close cooperation with PGC-1A and ERRγ (Villena and Kralli 2008). Crucially, BPA binds to ERRγ with high affinity (Okada et al. 2008), and ERβ has been identified as an important regulator of PPARγ (Foryst-Ludwig et al. 2008).

Given the structural homology between ERs and ERRs, particularly in the DNA-binding domain, involvement of ERRs in estrogenic signaling pathways is not unexpected (Giguère 2002). ERRα has been proposed as a regulator of aromatase activity (Yang et al. 1998), and in turn, estradiol induces up-regulation of ERRα in some tissues (Shigeta et al. 1997). ERRα stimulation of androgen-responsive element–containing promoters illustrates the potential for cross-talk with signaling driven by other steroid hormones (Teyssier et al. 2008).

The functional relevance of changes in ERβ and ERRα expression in blood leukocytes has not been determined. Because estrogens and androgens can exert differential effects in function depending on the cell type and its stage of development, the consequences

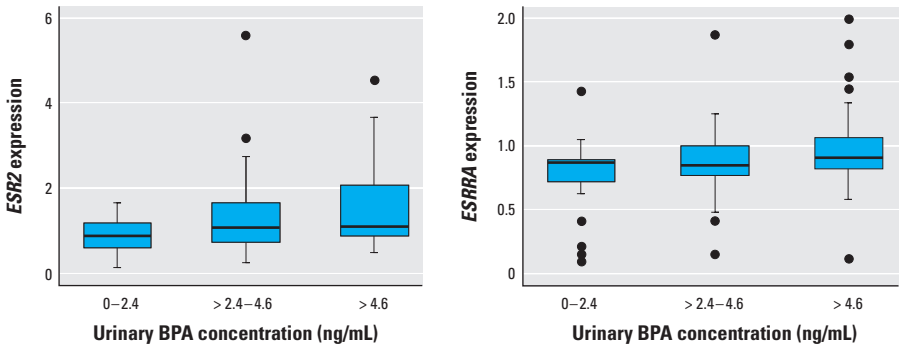


**Figure 1.** Box plot of *ESR2* and *ESRRA* probe intensity by urinary BPA concentration. Boxes extend from the 25th to the 75th percentile, horizontal bars represent the median, whiskers indicate the 10th and 90th percentiles, and outliers are represented as circles.

**Table 4.** Multiple regression model estimates for the associations between explanatory variables and natural logs of *ESR2* and *ESRRA* gene expression.

| | *ESR2* | | | *ESRRA* | | |
|---|---|---|---|---|---|---|
| Variable | Coefficient (95% CI) | *p*-Value | Std β | Coefficient (95% CI) | *p*-Value | Std β |
| BPA concentration (log transformed) | 0.1387 (0.001, 0.2764) | 0.048 | 0.1775 | 0.1886 (0.0324, 0.3448) | 0.019 | 0.2699 |
| Age | –0.0169 (–0.0261, –0.0078) | < 0.001 | –0.4256 | 0.0018 (–0.0086, 0.0122) | 0.733 | 0.0470 |
| BMI | 0.0206 (–0.0057, 0.0468) | 0.122 | 0.1399 | –0.0023 (–0.0312, 0.0265) | 0.873 | –0.0194 |
| Study site | 0.2016 (–0.0098, 0.413) | 0.061 | 0.1629 | –0.218 (–0.4489, 0.0129) | 0.064 | –0.2087 |
| Educational attainment | | | | | | |
| None/elementary | 1 | | | 1 | | |
| Secondary | 0.1146 (–0.2084, 0.4375) | 0.482 | 0.0834 | –0.4742 (–0.8521, –0.0962) | 0.015 | –0.4176 |
| High school | –0.0347 (–0.3704, 0.3011) | 0.838 | –0.0275 | –0.099 (–0.4865, 0.2885) | 0.612 | –0.0929 |
| Professional/university | 0.0899 (–0.2604, 0.4403) | 0.611 | 0.0542 | –0.0379 (–0.4521, 0.3764) | 0.856 | –0.0268 |
| LDL cholesterol (mg/dL) | –0.0024 (–0.006, 0.0012) | 0.195 | –0.117 | 0.0016 (–0.0022, 0.0055) | 0.407 | 0.094 |
| Triglycerides (mg/dL) | 0.0012 (–0.0002, 0.0025) | 0.095 | 0.1444 | –0.0009 (–0.0023, 0.0006) | 0.255 | –0.1339 |
| Neutrophil% | –0.3194 (–0.8618, 0.2229) | 0.245 | –5.1245 | 0.3622 (–0.2803, 1.0047) | 0.265 | 6.5463 |
| Lymphocyte% | –0.302 (–0.845, 0.2411) | 0.272 | –4.606 | 0.3611 (–0.2828, 1.0049) | 0.267 | 6.1452 |
| Monocyte% | –0.2982 (–0.8349, 0.2385) | 0.272 | –1.0051 | 0.3678 (–0.265, 1.0007) | 0.250 | 1.5245 |
| Eosinophil% | –0.3075 (–0.8726, 0.2575) | 0.282 | –0.8726 | 0.3624 (–0.2947, 1.0196) | 0.275 | 1.2623 |
| Constant | 31.078 (–23.088, 85.244) | 0.257 | | –36.1216 | 0.264 | 0 |

Std, standardized. "Constant" refers to the intercept term in the multiple regression model; it gives the expected log-transformed gene expression level when all other variables in the model are set to zero.

of BPA exposure on a wider range of adult reproductive and somatic tissues merits further attention (Goodman et al. 2008). However, up to 50% of expression changes in leukocytes for highly heritable *cis*-acting traits are also mirrored in other tissues such as adipose tissue, making them viable surrogates for exposure of other tissues (Emilsson et al. 2008). Human adipocytes express both ERβ and ERRα (Hugo et al. 2008), and adipocyte explants respond to both BPA and 17β-estradiol exposure in the nanomolar range by accumulating lipid. Taken together, these results are strongly suggestive of specific and targeted bioactivity of BPA *in vivo*, even if the clinical relevance, if any, of these findings is not yet clear.

One limitation of this analysis is its cross-sectional nature. Virtually all individuals are exposed, and because clinical trials to administer BPA in human subjects are ethically unacceptable, collecting longitudinal data demonstrating that BPA exposure induces gene expression changes *in vivo* is not currently achievable.

It is feasible that the increases in gene expression that we measured are associated with confounding variables that have not been accounted for in our models. For example, there are time-dependent changes in ERβ expression, both on a long-term scale, such as in fetal and postnatal development, and in short-term oscillations during the circadian cycle (Swedenborg et al. 2009). Although it is not possible to completely account for circadian cycles, all samples were taken at a similar time of day, and we restricted our analysis to men rather than women to minimize the influence of cyclic variation in endogenous hormones. Confounding variables that could affect BPA exposure include higher food intakes or obesity, which could be accompanied by incidentally higher intakes of BPA (Sharpe 2010). Our secondary analyses included adjustment for BMI and LDL cholesterol and triglyceride concentrations, and these made minimal difference to the overall results, arguing against obesity as an explanation for our findings. We found no associations between serum lipids and expression intensities of our candidate genes.

Another consideration is that we quantified BPA metabolites in urine, while gene expression was measured in blood leukocytes. BPA ingested in humans is rapidly excreted, so urine is considered the most viable biomonitoring approach for BPA, as detailed by Calafat et al. (2005). Single spot samples are limited measures of longer-term exposure, but a in study of temporal variability in urinary BPA metabolites, Mahalingaiah et al. (2008) found that a single spot sample had moderate sensitivity for predicting an individual's tertiary categorization. Nepomnaschy et al. (2009) measured stability of BPA over 2-week intervals in first voided urine samples from 60 women and found a Spearman correlation of 0.5, indicating that within-individual BPA exposures were generally stable over periods of weeks. They also showed that the stability of BPA in long-term frozen samples is good. The stability of free BPA in urine was also confirmed by Ye et al. (2011).

The mean BPA concentration in our study was 3.65 ng/mL, and assuming an average 24-hr urine volume of 1,600 mL in adult men (Galloway et al. 2010), a 100% excretion rate, and a total blood volume of 6 L, the estimated concentration of BPA in the blood was in the low nanogram per milliliter range. The *in vitro* $IC_{50}$ (half-maximal inhibitory concentration) for human ERβ receptor binding of BPA is in the micromolar range (Matthews et al. 2001), which would imply low ER occupancy rates. Given that functional effects of BPA on nuclear receptor expression have also been reported in both animal and human cells at this concentration, *in vitro* measurement may not be indicative of the *in vivo* situation where differential binding to carrier proteins and receptors may occur. There are no *in vivo* data on the rate at which BPA is converted to BPA-monoglucuronide and excreted from the body, only estimates, and because BPA is lipophilic with a log octanol–water partition coefficient ($K_{ow}$) of 2.2–3.82, distribution to lipid-rich tissues is a possibility. This suggestion is supported by population-based half-lives for BPA calculated by Stahlhut et al. (2009) to be significantly longer than previous predictions of 6 hr.

The major metabolite of BPA, BPA-monoglucuronide, has no estrogenic activity, but oxidative cleavage of BPA to form the estrogenically active metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) has been observed in rat liver. Okuda et al. (2010) reported that MBP was 500-fold more potent than BPA itself in inducing dose-dependent changes in expression of *ERα* and *ERβ* mRNA. The significance of this metabolite in humans is not yet known. However, a comparison of the phase 1 metabolism of BPA in rat and human liver microsomes identified the oxidation product BPA-catechol to be a minor (~ 10%) metabolite in both species. BPA-catechol is considered to be a weak estrogen (Nakagawa and Suzuki 2001), suggesting that further investigation of the phase 1 metabolism of BPA in humans and the estrogenic potency of all metabolites is merited (Ye et al. 2011).

## Conclusion

We provide the first report of associations between BPA exposure and *in vivo* estrogenic gene expression in humans. We examined *in vivo* expression of six ER, ERR, and AR genes in peripheral blood leukocytes from 96 adult men from the InCHIANTI population study. We observed positive associations between higher urinary BPA concentrations and higher expression of two estrogen-responsive genes, encoding ERβ and ERRα. The associations remained statistically significant when adjusted for potential confounders, including obesity and serum lipid concentrations. The individuals in the upper tertile of BPA exposure showed 65% higher mean expression of the *ESR2* (ERβ) gene in peripheral blood

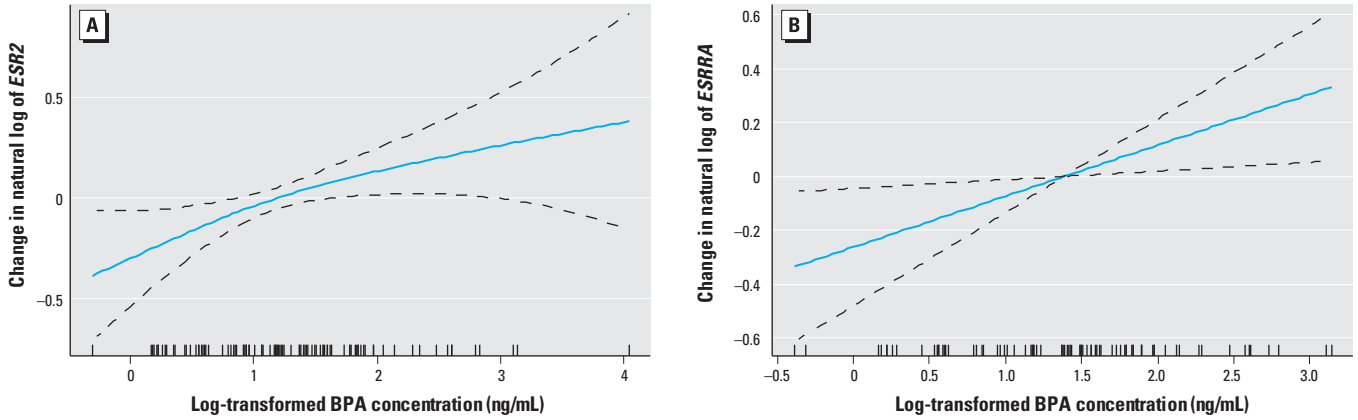


**Figure 2.** Cubic regression spline models illustrating the functional form of the relationship between log-transformed urinary BPA concentration and *ESR2* (*A*) and *ESSRA* (*B*) gene expression.

leukocytes than did those in the lower tertile. Although the clinical significance of these results is not yet known, such activation in humans provides evidence that BPA is likely to function as a xenoestrogen in this population-representative sample of adults. This prompts a need for replication and scientific follow-up, for example, in examining the relationship between gene expression changes and protein expression and effects of BPA exposure in a wider range of estrogen-regulated target tissues.

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# 4. Bisphenol A Modulates the Metabolic Regulator Estrogen-Related Receptor-α in T-Cells

*4.1 Statement of the candidate's contribution to the co-authored paper.*

The candidate was responsible for laboratory work, planning of the study, data analysis, and the drafting of the manuscript. These were carried out during the second and third year of the research programme. Study design, successive analysis and interpretation of the results, and the critical revision of the manuscript were discussed with Prof. T. Galloway, Dr. L. Harries, and Prof. D. Melzer.

# Bisphenol A Modulates the Metabolic Regulator Estrogen-Related Receptor-α in T-Cells

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## Abstract

Bisphenol A (BPA) is a widely used plastics constituent that has been associated with diabetes, cardiovascular disease and altered metabolic regulation. Evidence for how BPA exerts significant biological effects at chronic low levels of exposure has remained elusive. In adult men, exposure to BPA has been associated with higher expression of two nuclear receptors, estrogen-receptor beta (ERβ) and estrogen-related-receptor-alpha (ERRα), in peripheral white blood cells *in vivo*. Here we explore the expression of *ESR2* (ERβ) and *ESRRA* (ERRα) in human leukaemic T cell lymphoblasts (Jurkat cells) exposed to BPA and a BPA putative metabolite *in vitro*. We show that exposure to BPA led to enhanced expression of *ESRRA* within 6 hours of exposure (mean±SEM: 1.43±0.08 fold increase compared to the control, $p<0.05$). After 72h, expression of *ESRRA* remained significantly enhanced at concentrations of BPA ≥ 1nano molar (nM). Oxidative metabolism of BPA by rat liver S9 fractions yields the potent estrogenic metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP). Exposure of cells to 1-100nM MBP increased the expression of both *ESRRA* (significantly induced, $p<0.05$, at 1, 10, 100nM) and *ESR2* (1.32±0.07 fold increase at 100nM exposure, $p<0.01$). ERRα is a major control point for oxidative metabolism in many cell types, including T cells. Following exposure to both BPA and MBP, we found that cells showed a decrease in proliferation rate. Taken together, these results confirm the bioactivity of BPA against putative T cell targets *in vitro* at concentrations relevant to general human exposure.

**Key words:** Bisphenol A, endocrine disruption, estrogen receptor beta, estrogen-related receptor alpha, gene expression, T cells.

## List of any unusual abbreviations used in the manuscript:

B2M, beta 2 microgloubulin

BPA, bisphenol A

ESRRA (ERRα), estrogen related receptor α

ESR2 (ERβ), estrogen receptor β

$EE_2$, 17α-Ethinylestradiol

FAM102A, family with sequence similarity 102

FBS, fetal bovine serum

HK, housekeeping genes

HPRT1, hypoxanthine phosphoribosyl- transferase 1

LOD, limit of detection

MBP, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene

NMR, nuclear magnetic resonance

PPARγ, peroxisome proliferator-activated receptor-γ

RT-PCR, reverse transcription polymerase chain reaction

# 1. Introduction

Bisphenol A (BPA) is a synthetic compound originally synthesised as an alternative to estrogen (Dodds and Lawson, 1936). It is used widely as a monomer in polycarbonate plastics and in the epoxy resins that line food and drinks containers and is one of the world's highest production volume chemicals (Ritter, 2011). Leaching of BPA from food packaging into food and subsequent ingestion is a major route of human exposure, with additional exposures from dental sealants, inhalation of household dusts and dermal routes (Bailey and Hoekstra, 2011). This has led to ubiquitous exposure of the general population, with >95% of people showing detectable levels of BPA in their urine (Calafat et al., 2008; Galloway et al., 2010; Vandenberg et al., 2010).

There has been a level of controversy over the adverse health consequences of exposure to BPA. Recent studies have demonstrated that human and wildlife populations are exposed to levels of BPA capable of negatively impacting on reproductive, developmental and metabolic endpoints in different wildlife species and laboratory animal studies (Calafat et al., 2008; Vandenberg et al., 2010). In addition, an increasing number of epidemiological studies has now shown that general population exposure to BPA is associated with adverse health outcomes. BPA exposures were positively associated with endocrine changes in men (Galloway et al., 2010; Meeker et al., 2010) and with negative effects on immune function (Clayton et al., 2011). Positive prospective associations have been reported between BPA exposure and a range of reproductive and developmental conditions including recurrent miscarriage (Sugiura-Ogasawara et al., 2005) and externalising behaviour scores in the offspring of exposed mothers (Braun et al., 2011). Urinary BPA concentrations were associated with cardiovascular disease diagnosis in large scale cross-sectional (Lang et al., 2008; Melzer et al., 2012a; Melzer et al., 2010) and longitudinal studies (Melzer et al., 2012b).

The plausibility of these associations and a comprehensive understanding of the risks posed by exposure to BPA are complicated by a lack of understanding of the mechanisms underlying its varied endocrine disruptive actions. Many of the physiological effects of BPA have been studied in relation to its widely reported activity as an estrogen agonist (Chapin et al., 2008; Lee et al., 2003). A number of additional receptor-mediated effects have been reported including anti-androgen activity (Bonefeld-Jørgensen et al., 2007), thyroid hormone disruption (Moriyama et al., 2002), altered pancreatic beta-cell function (Ropero et al., 2008; Soriano et al., 2012) and obesity promoting effects (Marmugi et al., 2012; Newbold et al., 2007), binding to estrogen related receptor gamma (Okada et al., 2008; Takayanagi et al., 2006) and activation of peroxisome proliferator-activated receptor-γ mediated pathways (Kwintkiewicz et al.). Comprehensive reviews of the available data highlight both the complexity of BPA's biochemical and molecular interactions, and that these varied receptor systems and molecular pathways may be differentially affected in different target tissues and species (Thayer and Belcher, 2011; Wetherill et al., 2007).

In order to gain a better understanding of the molecular mechanisms underlying the health effects of BPA, we recently studied the *in vivo* expression of a panel of estrogen receptor-estrogen-related receptor and androgen receptor genes in the peripheral blood leukocytes taken from 96 adult men (Melzer et al., 2011). We found that there were positive associations between higher BPA concentrations and higher expression of 2 genes, *ESR2* (estrogen receptor 2, ERβ) and *ESRRA* (estrogen related receptor alpha, ERRα). This finding is of interest since it suggests that BPA and/or its metabolites are bioactive in the human body and that associations with hormone signalling and related disorders are biologically plausible. ERβ is important in mediating the physiological actions of estrogens in target tissues including prostate, colon, cardiovascular and central nervous systems (Imamov et al., 2005), with a tissue distribution and ligand specificity that is distinct from that of ERα. ERRα

belongs to the NR3β orphan nuclear receptor family subgroup, consisting of ERRα, β and γ. Although responsive to some synthetic estrogens, there are no known naturally occurring ligands for ERRα (Willy et al., 2004). Genetic studies in mice show that ERR subtypes are involved in mitochondrial biogenesis, oxidative phosphorylation and lipid metabolism (Tremblay and Giguere, 2007). Both ERRα and γ are essential for proper cardiac function (Huss et al., 2007) and may play a broader role in metabolic homeostasis (Tennessen et al., 2011). The functional relevance of changes in ERβ and ERRα in blood leukocytes has not been comprehensively determined, although ERRα is reported to be a selective transcriptional regulator of certain T cell effector functions, facilitating gene expression and glucose and mitochondrial metabolism during T cell growth and proliferation (Michalek et al., 2011).

The objectives of the current study were to explore the expression of the *ESR2* and *ESRRA* genes in human leukaemic T cell lymphoblasts (Jurkat cells) exposed to BPA *in vitro.* In addition, we investigated the effect of a putative metabolite of BPA: oxidative metabolism of BPA by rat or human liver S9 fractions yields the potent estrogenic metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), which has around 1000 fold higher estrogenic activity than BPA (Okuda et al., 2010). 3D models of MBP suggest that its structure allows for interaction with amino acid residues in ERα and ERβ that are important for binding of estradiol by these receptors (Baker and Chandsawangbhuwana, 2012) We compared the expression of ERβ and ERRα following exposure to 17α-Ethinylestradiol ($EE_2$), BPA and MBP at environmentally relevant exposure concentrations and over different time intervals. Since ERRα is a major control point for oxidative metabolism in many cell types, including T cells, we determined the effects of BPA and MBP exposure on the downstream end-point of cell proliferation.

## 2. Material and Methods

**2.1 Synthesis of 4-methyl-2,4-bis (p-hydroxyphenyl) pent-1-ene (MBP).** MBP was synthesized at Hiroshima International University as previously described (Yoshihara et al., 2004). MBP was in the form of a white powder with melting point 130.5-132ºC; purity was certified from the narrow melting point range obtained and the single peak obtained by HPLC. The $^{1}H$ NMR data (400MHz, $CDCl_3$) of authentic MBP obtained using a JEOL, ALPHA-FT NMR Spectrometer,was: δ 1.20 (s,6H), 2.74 (s,2H), 4.64 (s, 1H), 4.71 (d, 1H,J = 2.0Hz), 4.74 (s,1H), 5.07 (d,1H, J= 2.0Hz), 6.67(d,2H, J=8.8Hz),6.67(d,2H, J =8.4Hz), 7.09 (d,2H, J =8.8Hz), 7.12 (d,2H, J =8.8Hz). Chemical shifts are reported in ppm downfield from the peak of tetramethyl-silane (TMS) used as an internal standard. Splitting patterns are designated as "s and d" indicating "singlet and doublet", respectively.

**2.2 Cell culture, dosing and exposure.** All further experiments were conducted at the University of Exeter**.** Jurkat cells, human leukaemic T cell lymphoblasts, were purchased (catalogue number: 88042803) and cultured in RPMI-1640 without phenol red (Sigma-Aldrich Company Ltd., UK), supplemented with 10% fetal bovine serum, FBS, stripped with charcoal-dextran (Gibco, Gran Island, NY) and 200mM L-glutamine (Sigma-Aldrich). Jurkat cells were considered a good model system for several reasons. Firstly, our population level study (Melzer et al., 2011) was carried out on whole blood, therefore the choice of a lymphocyte cell line in which to investigate our findings further seems appropriate. Secondly, BPA exposure is known to have effects on cytokine production in T-cells in vitro (Yan et al., 2008). Finally, ERRα, has many functions, including modulation of the immune response (Ranhotra, 2012). Cells were grown in suspensions at 37ºC- 5% $CO_2$ and culture was maintained between 0.5-5 x 100.000 cells/ml. Before the experiments, cells were grown in a

single tissue culture flask, angled neck vent cap, and then used for the experiment at passage 3. Medium was changed ahead of the start of each experiment and it was not changed during the treatments. Cell viability was assayed with Trypan blue (Sigma-Aldrich) staining (dilution: 1/10th of 100µl culture volume). Cell viability was always above 80%. Cells were exposed to 17α-Ethinylestradiol ($EE_2$; ≥98%, Sigma-Aldrich), Bisphenol A (BPA; ≥99%, Sigma-Aldrich) and to the oxidative metabolite of BPA, 4-methyl-2,4-bis (p-hydroxyphenyl) pent-1-ene (MBP), synthesised as described above.

**2.3 Exposure to $EE_2$**. Cells were equally divided into 36 small flasks (25 $cm^2$) and each flask was considered as a single sample. Each flask was assigned to either the control or the exposed group: 1 µM $EE_2$ and the solvent control (0.001% ethanol) were added accordingly. Total RNA extraction was performed at time point 0 hour, 6 hours (hrs), 24hrs, 36hrs, 48hrs, 72hrs. The choice of these time points aimed at providing a very comprehensive description of the time dependent activation of the target genes across a time interval of 3 days. The selection of a time window of 72 hours was based on previous studies present in the literature (Naciff et al., 2010; Richter et al., 2007). During each time point 3 flasks from the control and the exposed group were randomly selected, sampled for RNA extraction and then discarded.

**2.4 Exposure to BPA and MBP**. Jurkat cells were divided into treatment and control flasks, 1µM BPA and solvent control (0.001% ethanol) were added accordingly. Cells were then seeded onto 24-well plates. Total RNA extraction was performed at time point 0 hour, 6hrs, 24hrs, 36hrs, 48hrs, 72hrs. When exposures of 1 nM, 10 nM and 100nM BPA concentrations were then tested, mRNA expression was measured at a single time-point (72hrs). The 72hrs time-point was considered a sensitive window based on the results of the 1µM BPA experiment. Treatments and solvent controls were added accordingly to the different concentrations as previously described. Treatments were replicated in 8 wells per experiment and the control in 6. Two parallel experiments were conducted; results were checked for

statistical differences, then tabulated and merged. Similarly, a set of experiments was performed exposing cells to MBP that was tested at 1nM, 10 nM, 100nM concentrations. Study design, replication and sampling time were as the ones described above.

**2.5 Real time RT-PCR measurement of gene expression in target genes.** Total RNA was extracted after sampling using the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. RNA yield was quantified using a NanoDrop spectrophotometer. Total RNA (500ng) was reversed transcribed in 20µl reactions using the Superscript III VILO kit (Invitrogen, Paisley, UK). Real time PCR amplifications were performed on the ABI 7900HT platform using 96-well plates (Applied Biosystems, Foster City, USA). Each well contained 5µl TaqMan 2x universal master mix (no AMPerase) (Applied Biosystems, Foster City, USA), 3.5µl $dH_2O$, 1µl cDNA template and 0.5µl of probe. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression levels of the genes *ESR2* (estrogen receptor 2; ER beta; ERβ), and *ESRRA* (estrogen related receptor alpha; ERRα) were assessed through relative quantification using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data are presented as the fold change in gene expression relative to 3 endogenous control transcripts and normalised to levels of the test transcripts in the untreated control. PCR signal of the target transcripts was standardized using three housekeeping (HK) genes: *B2M* (β2 microgloubulin), *HPRT1* (hypoxanthine phosphoribosyl-transferase 1) and *FAM102A* (family with sequence similarity 102). This was done to account for differences in the amount of cDNA that is loaded into each PCR reaction wells and differences between plates. The expression of each of the five genes was measured in triplicate for each sample: this technical procedure provides a more accurate PCR analysis, for it helps removing technical errors or highlights weak and variable signals. A minimum of three HK genes is recommended for a reliable standardization factor and to reduce errors in real-time quantitative RT-PCR data

(Vandesompele et al., 2002). *B2M* and *HPRT1* are widely used HK genes (Vandesompele et al., 2002) while *FAM102A* expression was shown empirically not to be influenced by BPA exposure in our previous experiments. Probes were inventoried Applied Biosystems assays, with the exception of the *ESRRA* probe, which was a custom assay (probe and primer sequences available on request).

**2.6 Cell count.** Cell count was carried out using the particle counting Multisizer Coulter Counter (Beckman, USA).

**2.7 Statistical analysis.** Values in tables and graphs are expressed as mean ± SEM (unless otherwise specified). Two-way factorial ANOVA was used to test the interaction between treatment and time of exposure when multiple time-points were considered. Data were checked for the normality of distribution and variance homogeneity. Where the overall ANOVA showed significance, post-hoc comparisons between treatment and its control were performed. When only the effect of different treatments was tested, one-way ANOVA was used followed by Bonferroni post-hoc test. *p*-value $<0.05$ was considered significant.

## 3. Results

**3.1 Time and dose responsiveness of ESR2 and ESRRA in Jurkat cells.** All target genes (*ESR2, ESRRA*) and HK genes (*B2M, HPRT1, FAM102*) were empirically checked and consistently detected in Jurkat cells. The estrogen responsiveness of cells was initially tested using 17α-Ethinylestradiol ($EE_2$) (Naciff et al., 2010). A time dependent increase in expression of both *ESR2* and *ESRRA* target gene transcripts was seen (Figure 1A and 1B) following exposure to 1μM $EE_2$. For *ESR2*, a monotonic increase was seen, with maximum intensity of expression at 72h and indication of an increasing trend (Figure 1A). On the other hand, *ESRRA* showed a biphasic trend with a non-significant increase in gene expression after 6 hours, a subsequent drop, and a significant increase at 48 and 72 hours (mean±SEM, up to

2-fold increase +/- 0.09 compared to the control, $p<0.05$). Exposure to 1μM BPA under the same conditions produced a similar level of increase (Figure 1C and 1D). The time profile of BPA exposure indicates that *ESR2* expression was significantly induced compared to the control after 36 hours (1.36±0.10 fold increase, $p<0.05$). Expression of *ESRRA* again showed a biphasic response: a statistically significant increase was registered after only 6 hours of exposure to BPA followed by a drop at 24 hours and a second significant increase at 48 hours (1.58±0.08 fold increase, $p<0.01$ remaining elevated for the duration of the experiment. Overall, exposure to a high concentration of BPA showed a stronger and quicker increase in mRNA expression levels of *ESRRA* than *ESR2*.

Exposures were then conducted at environmentally relevant concentrations (1nm – 100nm) of BPA (Figure 2A-B). Similar concentrations were selected in the MBP experiments (Figure 2C-D). The *ESR2* mRNA expression level did not change in treatment groups exposed to increasing concentrations of BPA (Figure 2A). There was however a significant increase in *ESR2* expression following 72h exposure to 100nM MBP: the average value in the treatment group (mean± SEM, 1.32±0.075, n=16) was significantly higher than the control group (1.01±0.046, n=12, Figure 2C). For *ESRRA*, significantly increased expression was seen following exposure to both BPA and MBP at all concentrations from 1nM upwards (Figure 2B and 2D). Jurkat cells exposed to increasing concentrations of BPA showed levels of *ESRRA* expression increased by up to 82% (± 13%, n=16). Similar results were registered after exposure to MBP (Figure 2C and D).

**3.2 Effects of BPA and MBP on cell proliferation.** Jurkat cells exposed to 100 nM BPA showed a decreased number after 48 hours compared to the control (Figure 3A). After 72 hours, the difference in cell count between exposed and control groups was greater ($p<0.001$). Similarly, Jurkat cells exposed to 100 nM MBP showed a decreased proliferation after 48 and 72 hours (Figure 3B).

## 4. Discussion

We have previously reported that human exposure to BPA is associated with the activation of the estrogen responsive genes *ESR2* and *ESRRA* measured in peripheral blood leukocytes in the general adult population (Melzer et al., 2011). The ERs and ERRs share common targets and control overlapping transcriptional regulatory networks. This makes them of high interest for investigating the effects of BPA and related compounds showing diverse, estrogen-like effects. The aim of this study was to further explore the expression of *ESR2* and *ESRRA* in human leukaemic T cell lymphoblasts exposed to BPA under laboratory conditions where potentially confounding variables could be controlled. We show here that BPA and the oxidative derivative MBPat environmentally relevant concentrations can cause changes to hormone sensitive gene transcripts and that this has physiological consequences in altering cell proliferation rate in T cells. This is important because these results, taken together with our previous study, suggest that *ESRRA* gene expression can be significantly modulated by BPA in human blood cells, both in laboratory and *in vivo* conditions; hence, imply that BPA is capable of plausible bioactivity in the human body.

Of particular interest is what we believe to be the first report of a significant increase in expression of *ESRRA* following exposure to both $EE_2$ and BPA in T cells. ERRα is a regulator of cell metabolism across many different cell types (Giguère, 2008; Villena and Kralli, 2008) and has a central role in regulating energy homeostasis and adaptive oxygen capacity. Activity of ERRα, which has no known endogenous ligands, is known to be regulated at the protein level; activity is highly sensitive to co-regulatory proteins involved in energy homeostasis such as the steroid receptor co-activator (SRC), peroxisome proliferation activation receptor gamma co-activators (PGC)-1α/β and the co-repressor receptor-interacting

protein 140 (RIP140). Our data show additional regulation at the level of the messenger RNA, indicating both the importance of transcriptional regulation (Giguère, 2008) and highlighting a novel target for the action of endocrine disrupting chemicals in the body.

The activity of ERRα as a transcriptional regulator in T cells has recently been described in detail (Michalek et al., 2011). T-cell activation and differentiation are energetically demanding processes and considerable metabolic reprogramming is required to enable the rapid proliferation and functional responsiveness of T effector (Teff) and repressor (Treg) cell subsets. Accordingly, ERRα appears to play a key role in Teff cell function through the induction of metabolic gene expression to programme mitochondria for aerobic glycolysis and to regulate glycolytic metabolism more generally. Interference in this process by BPA could in theory alter the balance of Teff and Treg cells, leading to immunotoxic effects. Although the full significance of ERRα expression in the Jurkat lymphoblastic cell line that we have used here is not yet known, we were able to show a significant inhibition of cell proliferation following exposure to 100nm BPA, consistent with this theory.

There may be some factors to consider when interpreting this result. Given the fact that these were not primary cells, senescence should not affect Jurkat T cells. Cell viability was checked before the experiment using Trypan blue assay that is a dye exclusion test used to determine the number of viable cells present in a cell suspension. Yet, we did not perform an analysis relative on the cell cycle arrest which could be an early sign of cell apoptosis.

It would be of considerable interest to determine the effects of BPA on *ESRRA* expression and metabolic activity in a wider range of different cell types, especially in light of the associations previously reported between exposure to BPA and metabolic disease (Melzer et al., 2012a; Melzer et al., 2012b). ERRα is present in a wide variety of cell types and is highly expressed in cells with elevated metabolic demand such as heart, skeletal muscle and brown

adipose tissue tissues (Eichner and Giguère, 2011). It is suggestive to note that BPA has shown affinity for the metabolic regulator peroxisome proliferator-activated receptor-γ (PPARγ) in a previous study (Kwintkiewicz et al., 2010) and in addition there is evidence of agonist interaction between PPARγ and ERRα (Willy et al., 2004) and PPARγ and ERβ (Foryst-Ludwig et al., 2008). More recently, Marmugi et al (2012) used a transcriptomic approach to show that exposure of male CD1 mice to low doses of BPA was able to influence de novo fatty acid synthesis through alterations in lipogenic genes. These results suggest that the role of BPA in influencing metabolic processes is certainly deserving of further attention.

BPA metabolism and excretion are believed to take place largely in the first 24hrs after exposure, although there are studies suggesting this could take longer (Christensen et al., 2012; Sieli et al., 2011; Stahlhut et al., 2009). After liver metabolism, glucuronide conjugated BPA shows little or no affinity for nuclear receptors (Matthews et al., 2001). In human biomonitoring studies, analyses performed on blood, serum, or plasma samples measured unconjugated BPA which is considered the portion of BPA not yet metabolised by the liver and so capable of bioactivity. A recent review of these studies indicates that the range of blood/serum BPA concentrations spans between 0.5 and 2.5 ng/ml (Vandenberg et al., 2010). Given the range of concentrations chosen for our experiments (1-10nM; 1nM BPA = 0.228 ng/ml), we could argue that this reflects very well the physiological internal exposure to unconjugated BPA. The same range of concentration has been taken as reference in other major experimental studies (Hugo et al., 2008; Richter et al., 2007), whilst a recent review of human toxicokinetic studies suggests the circulating concentration may be lower than this (Doerge and Fisher, 2011).

Oxidative cleavage of BPA to form the estrogenically active metabolite MBP was first reported in rat liver and Okuda (Okuda et al., 2010) reported that MBP was 500 fold more potent than BPA in inducing dose –dependent changes in expression of *ESR1* and *ESR2* mRNA. Here, we have shown a small but significant up-regulation of ERβ following exposure of cells to 100nM MBP, which would agree with an enhanced estrogenic activity of this compound. In addition, MBP showed a near identical pattern of up-regulation of *ESRRA*, with a significant increase in expression seen at concentrations as low as 1nM. The full significance of this metabolite in humans is not yet known. MBP is formed by recombination of the radical fragment of BPA, and its formation requires both microsomal and cytosolic fractions. A similar mechanism of metabolism has been reported in sewage sludge bacterial isolates (Spivack et al., 1994). Whilst the glucuronate/sulphate conjugates are the main metabolites excreted in urine, a comparison of phase 1 metabolism in human and rat liver microsomes has identified the oxidation product BPA-catechol as a minor metabolite (approximately 10%) in both species, and BPA-catechol is certainly measurable in human urine (Ye et al., 2011). Since BPA-catechol itself is also reported to possess estrogenic activity, further investigation of the pathways of BPA metabolism and related compounds in humans would seem to be a priority for future research.

## 5. Conclusions

We provide here a first report of the enhanced expression of two estrogen responsive genes, *ESR2* (ERβ) and *ESRRA* (ERRα) in human leukaemic T cell lymphoblasts exposed to BPA at concentrations relevant to general population exposure. Significantly enhanced expression of both of these transcription factors *in vivo* has previously been associated with exposure to BPA in peripheral white blood cells in adult men (Melzer et al., 2011). Since ERRα is a major control point for oxidative metabolism in many cell types, including T cells, we also

determined the effects of BPA and MBP exposure on cell proliferation and showed a significant inhibition at nanomolar concentrations of both compounds. These results add to the weight of evidence for how BPA is able to exert significant biological effects at chronic low levels of exposure and identify a novel metabolic target for the action of endocrine disrupting chemicals.

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**Figure 1.** Gene expression intensity as a function of time following exposure of Jurkat cells to 1µM $EE_2$ (*A* and *B*) or 1µM BPA (*C* and *D*). Values are expressed as fold-change increase compared to each time-point control (mean ± SEM). The number of replicates is shown in each bar (* when $p<0.05$).

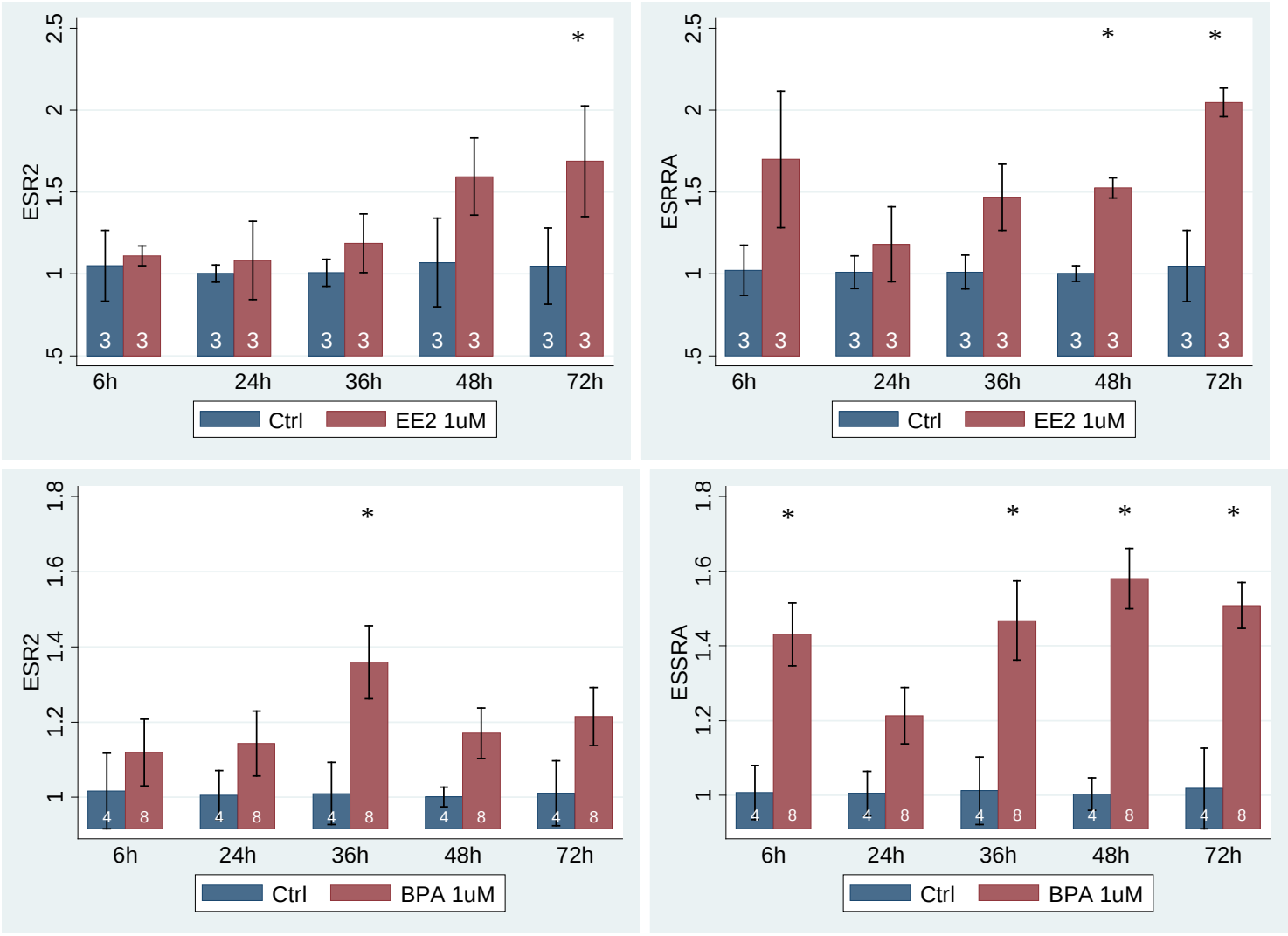


**Figure 2.** Gene expression intensity after 72 hours exposure of Jurkat cells to 1nM-10nM-100nM BPA (*A* and *B*) or 1nM-10nM-100nM MBP (*C* and *D*). Values are expressed as fold-change increase compared to control (Ctrl). Box plots follow Tukey's rules and outliers are represented by dots (see D).Number in brackets is the number of replicates. (* when $p<0.05$)

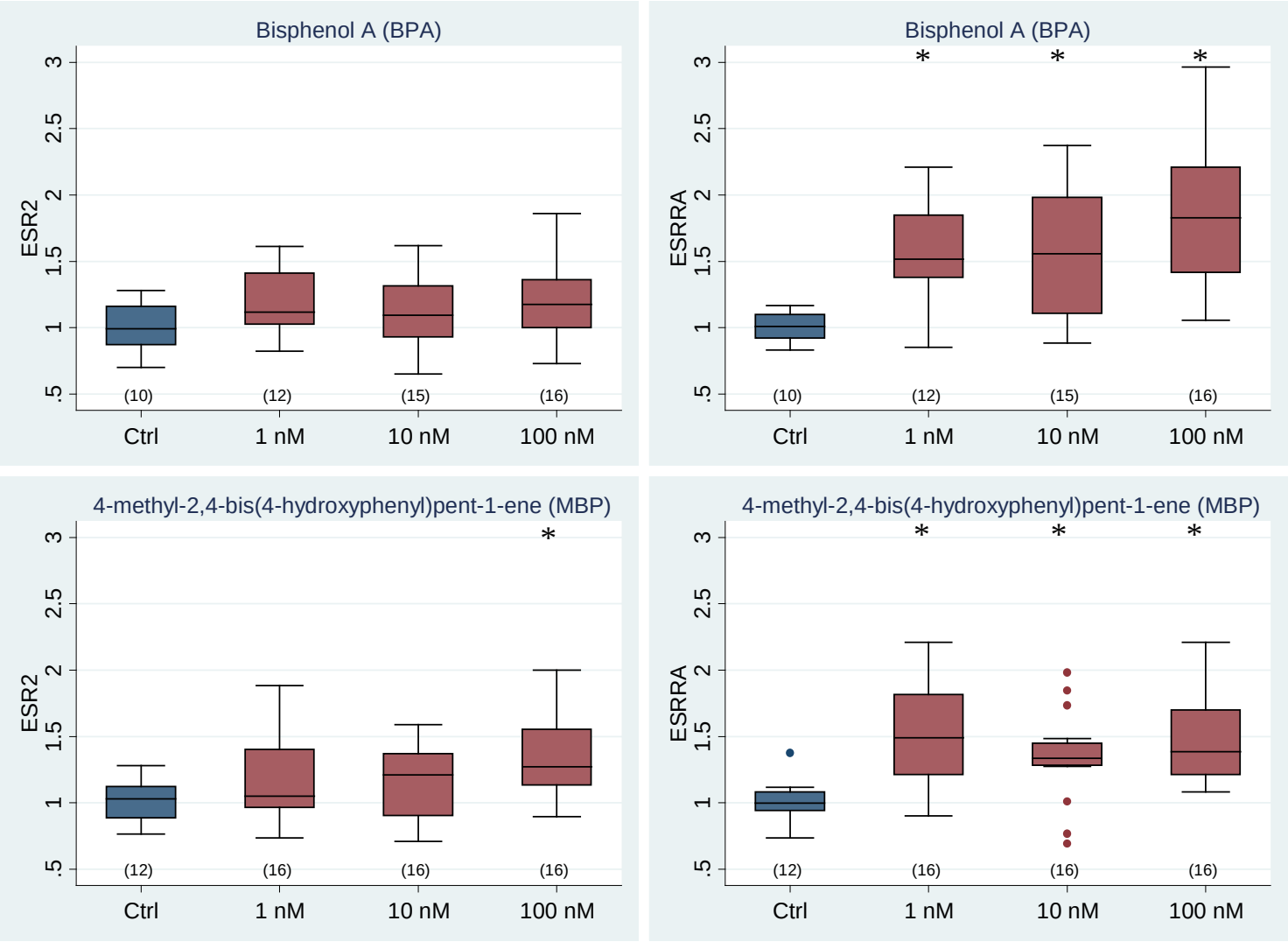
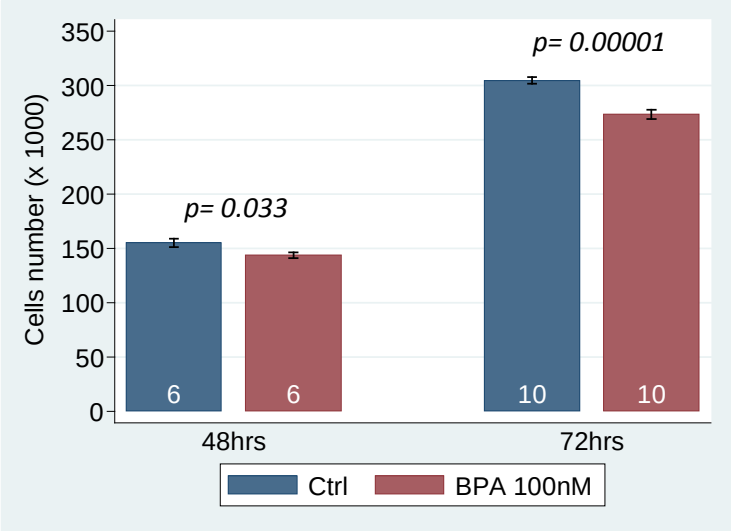
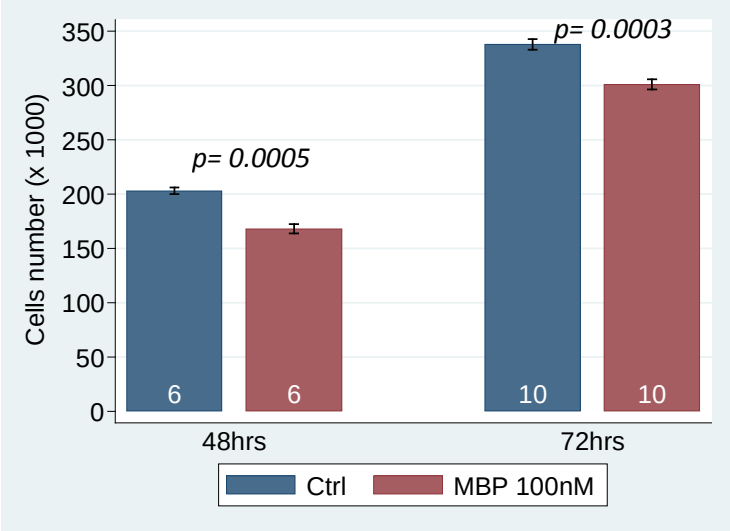


**Figure 3.** Proliferation rate decrease (*A* and *B*) in Jurkat cells exposed to 100nM BPA and 100nM MBP after 48 and 72 hours compared to control. Values are expressed as mean±SEM. The number of replicates is shown in each bar.. *p*-values are reported above the bars when $p<0.05$.



# 5. Long Term Stability of Urinary Bisphenol A Concentrations in a European Adult Population

*5.1 Statement of the candidate's contribution to the co-authored paper.*

The candidate had full access to all of the data in the study and was responsible for the statistical analysis: descriptive statistics, data tabulation, analyses of the association between uBPA concentration and parameters in the InChianti dataset, drafting of the manuscript were carried out during the second and third year of the research programme. Analysis and interpretation of the results were discussed with Prof. T. Galloway and Prof. D. Melzer who were responsible for the study design, the accuracy of the data analysis, and the drafting and critical revision of the paper.

# Long Term Stability of Urinary Bisphenol A Concentrations in a European Adult Population

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**Running title:** Bisphenol A exposure across a nine year interval.

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## Abstract

Bisphenol A (BPA) is a high production volume chemical with widespread exposure. Epidemiologic studies suggest associations with health outcomes, although these largely rely on urinary BPA (uBPA) concentrations measured at single time points. Little is known about the variability of uBPA concentrations over long time periods. Here we aim to compare uBPA concentrations measured in 218 participants (aged 32-75 years) of the InCHIANTI longitudinal study at baseline (1998-2000) and nine years later (2007-09), and to identify characteristics associated with uBPA concentrations.

Geometric mean baseline uBPA was 3.87 ng/ml (range 0.53 to 20.78 ng/ml) and slightly lower nine years later (3.17 ng/ml, range <0.5 to 29.63 ng/ml). Within subject logged uBPA concentrations were correlated ($r$=0.58; $p$=0.013, model adjusted for age, sex, urinary creatinine). Subjects in the top quartile ($4^{th}$) of uBPA distribution at baseline were, nine years later, still in the top $3^{rd}$ or $4^{th}$ quartile in 70% of cases. Among possible predictors of BPA exposure, age and alcohol consumption were the two most consistently associated to uBPA throughout the period investigated.

These results show that uBPA concentrations can be relatively stable over lengthy periods, and hence adds to the plausibility that reported associations with health outcomes may be causal.

**Key words:** bisphenol A, endocrine disruption, human biomonitoring, health effects, inchianti study, stability.

## Introduction

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide with an annual production of 8 billion pounds. Used as a plastic monomer and plasticizer, BPA is found in numerous consumer products and a number of studies have detected BPA leaching into foods (1). Since BPA has demonstrated endocrine-modulating ability in laboratory and population studies, there are concerns that the amount of BPA to which humans are exposed may cause adverse health effects.

There are now a number of studies showing associations between exposure to BPA and health conditions in the general population (2-7). Using data from the U.S. National Health and Nutrition Examination Survey (NHANES) for 2003–2004 and 2005–2006, we showed for the first time an association between BPA exposure and cardiovascular disease, type 2 diabetes in people aged 18 to 74 years old (8, 9). Similarly, our recent longitudinal study in healthy adults of the Norfolk-EPIC study followed for up to 10 years showed that higher uBPA concentrations predicted coronary artery disease onset (10).

The results from these and other human biomonitoring studies have attracted the attention of regulatory agencies. The correct estimation and the association of human exposure to BPA and related health outcomes are subject of debate (11). Currently, the matrix of choice for such studies is urine, since orally ingested BPA is rapidly metabolised via hepatic glucuronidation and excreted via the kidneys. Spot samples remain the most practical method of choice for large scale biomonitoring and are the regulatory methods of choice (12). However, there remain knowledge gaps in our understanding of how a single spot urine sample may be representative of chronic exposures. Due to the short biological half-life of BPA, it has been suggested that only

24 hours urine measurements could account for considerable within-day variation in subjects.

Moreover, given the long latency period of the health outcomes associated with BPA exposure, such as cardiovascular disease and diabetes, any measure of exposure should be reasonably stable over several years, or perhaps decades.

A few studies on temporal stability of BPA exposure have been published but focused on intervals of days or a few weeks (13-19). To the best of our knowledge, no longitudinal studies on a general population have been designed to study long term variability of uBPA over long time periods.

Given these uncertainties, we set out to explore the temporal stability of urinary BPA concentrations measured across longer time intervals (9 years). We recently published a new study on a European population, the InChianti longitudinal study (5), in which at baseline (1998-2000) participants were requested to collect urine for 24 hours; this was the first study to address questions on BPA measurement methods and the 24h exposure to BPA. Also, participants completed a questionnaire on daily food intake: food is considered a primary source of BPA exposure and changes in diet are believed to be reflected in variations of uBPA levels.

Here we present data on concentrations of uBPA measured in 218 subjects (aged 32 to 75) who took part in both the baseline study and the 9 year follow-up. Participants provided a 24 hour urine specimen at baseline (1998-2000) and a single-spot specimen at follow-up (2007-09). Therefore, we aimed to provide indications of changes in BPA exposure in a general population along a 9-years time window. We also examined the association between uBPA concentrations and possible predictors such as age, sex, BMI, socio-economic condition, and alcohol consumption. Because BPA is often used as a monomer in epoxy resins that are used to line food and beverage containers, we

investigated whether changes in uBPA concentrations across the period considered were associated with changes in canned food and soda drinks consumption.

## Materials and Methods

***Study population***. The InCHIANTI study (http://www.inchiantistudy.net/study.html) was designed to identify risk factors for mid- and late-life morbidity and was conducted in the Chianti area (Tuscany) of Italy. Participants were originally enrolled in 1998-2000 (Baseline), and consented to collect urine for 24 hours. Subjects were then seen every three years and provided single-spot urine specimens. Levels of 24h uBPA were measured at baseline in 720 adults (aged 20 through 74 years), a subset of the full cohort. Details have been previously described (5). The recent 9-year follow-up exam ($3^{rd}$ wave) was carried out in 2007-09: we analysed uBPA concentrations in 264 subjects selected randomly between the 309 participants (aged 75 or younger) who consented to give urine specimen (Table 1). The number of subjects with uBPA concentrations measured both at baseline and $3^{rd}$ wave was 220. Two subject with extremely high value of uBPA (>50 ng/ml) in the $3^{rd}$ wave were dropped, this left 218 participants.

***Analysis of urinary BPA concentrations.*** Analysis of samples was performed at the Brixham Environmental Laboratory Division of Analytical Chemistry (a division of AstraZeneca PLC) in compliance with Good Laboratory Practice, EU Directive 88/32/EEC. To measure total (free and conjugated) urinary concentrations of BPA, we used the methods employed by NHANES and adopted by the Division of Environmental Health Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention to prepare the urine samples (20). Analyses where carried out using online solid-phase extraction (SPE) coupled with high-

performance liquid chromatography (LC)–isotope dilution tandem mass spectrometry (MS/MS) with peak focusing. This has been described more extensively elsewhere (5). The limit of detection (LOD) was 0.5 ng/ml. At Baseline, no observation was below LOD; in 2007-09, two observations were <LOD and a value of 0.28 ng/ml was assigned instead, as in our previous studies (8, 9). In 2007-09, uBPA concentrations were measured in single-spot urine samples: in order to account for urine dilution, we used creatinine adjustment and measures of urine specific gravity (SG). Urinary creatinine is often used to adjust for urine dilution in single-spot uBPA measurement (20). Similarly, SG has been used in other studies as an alternative to creatinine correction (3, 14). Since baseline uBPA measurements were based on 24hrs urine collection, no urine dilution adjustment was considered when we analyzed baseline data.

***BPA potential exposure predictors and demographic covariates***. Participants received a food questionnaire originally developed and validated in the European Prospective Investigation into Cancer (EPIC) and were followed by trained interviewers (21). Initially, analysis on the association between uBPA concentration and single food items consumption was carried out at baseline where 300 variables were available. The same was done in the 3$^{rd}$ wave. This initial approach resulted in two major problems: the necessary multiple testing adjustments allowed comparison only with very low p-values; although extensive, the questionnaire provided little information on food packaging, or any other characteristics that could link food to potential BPA exposure through contact, hindering the interpretation of the results. For this reason, in addiction to common demographic covariates, we decided to include in our analysis only food variables which could be related to BPA exposure through packaging contact. Since BPA is often used as a monomer in epoxy resins that are used to line food and beverage containers (22), we considered information on canned meat and canned fish

consumption (frequency of canned meat/fish eaten per day). Both variables were analyzed and then considered together as "canned food" consumption. Similarly, carbonate, isotonic and soda drinks consumption (frequency per day) was included in the analyses because often linked to BPA exposure (23). Participants declared how many time per day they drink wine, beer and spirits. This information was summed into one single variable defined as alcohol consumption. Alcohol consumption was calculated as frequency of drinks per day. Participants were requested to define annual income as "adequate", "barely adequate", or "inadequate". Smoking habits were classified as "never", "former", and "current" smoker. BMI was tested as a continuous variable and as a categorized variable with subjects divided in underweight (< 18.5 kg/m2), recommended weight (18.5–24.9 kg/m2), overweight (25.0–29.9 kg/m2), obese I (30.0–34.9 kg/m2), and obese II (≥ 35 kg/m2) categories.

***Statistical analyses.*** Descriptive statistics of uBPA concentrations from baseline and 3$^{rd}$ wave analyses were tabulated. Correlation with demographic variables was tested and graphically examined. Because uBPA has a skewed distribution, values were log transformed in regression analyses. Linear regression models were adjusted for age and sex and then separately for creatinine and SG. Because baseline uBPA was measured in 24-hours collection samples, creatinine and SG adjustment was considered appropriate only for single-spot uBPA (3$^{rd}$ wave). Creatinine measurements were available for 186 specimens whereas SG levels were available for 214. Spearman correlation was calculated between differences in uBPA across the two analyses and differences in BMI, income, canned food intake, alcohol and soft drinks consumption from 1998 to 2007.

We carried out a set of sensitivity analysis to test the robustness of our findings, and correlation between uBPA measurements was reassessed considering changes in alcohol consumption and other predictors of uBPA concentrations, from 1998-00 to 2007-09.

## Results

Urinary BPA concentration was measured in samples from the InCHIANTI baseline (1998-2000) when subjects were requested to collect the urine produced in 24 hours. Single-spot uBPA concentrations were then measured approximately nine years later, during the 3rd wave of the study (2007-09). Demographic characteristics of the participants are summarized in Table 1. The age (mean ± SD) at the baseline was 48.9 ± 14.4 years; there were equal numbers of males (109) and females (109). Baseline and 3rd wave uBPA concentrations are presented in Table 2. At baseline, geometric mean uBPA was 3.87 ng/ml (95% CI 3.57 to 4.19). The distribution was skewed, with a 10th percentile of 1.9 ng/ml, a median of 3.8 ng/ml, and a 90th percentile of 7.9 ng/ml. In 2007-09, geometric mean uBPA was 3.17 ng/ml (95% CI 2.84 to 3.55). BPA distribution remained skewed, with a 10th percentile of 1.2 ng/ml, a median of 2.96 ng/ml, and a 90th percentile of 9.6 ng/ml. In Table 2, age and gender difference appear as predictors of uBPA levels. Levels of uBPA were higher in men than women in 1998-00 and 2007-09. Urinary BPA concentrations followed a clear negative age trend both at baseline and 3rd wave (Figure 1).

***Stability over 9 years.*** Baseline logged uBPA concentrations were significantly correlated with the 9-year follow-up measurements in a linear regression model adjusted for age and sex (n=218; $r$ =0.40; β=0.3040, 95% C.I. 0.1354-0.4726; $p$<0.001). In age, sex and urinary creatinine adjusted models, associations between uBPA measurements increased (n=186; $r$=0.57; β=0.2063, 95% C.I. 0.0411-0.3716; $p$=0.015). A third model,

adjusted to age, sex and urine specific gravity (SG) provided no substantive changes compared to the previous model (n=214; $r$=0.59; β=0.1784, 95% C.I. 0.0216-0.3352; $p$=0.026). In Figure 2, we show the log values scatter plot of baseline and 3rd wave uBPA measurements.

We also examined associations between uBPA baseline and 3rd wave quartile distributions: overall, subjects classified in the top quartile (4th) of uBPA distribution at baseline were, nine years later, still in the top 3rd or 4th quartile in 70% of the cases. Similarly, the proportion of subjects in the 1st quartile at baseline that were still in the 1st or 2nd quartile nine years later was 65%.

***Factors associated with changes in uBPA concentrations.*** We then investigated whether participants with large differences in uBPA from baseline to follow-up also reported changes in possible predictors of uBPA exposure. Initially, main covariates such as BMI, socio-economic condition, alcohol consumption, canned food and soft drink consumption were fitted in regression models to test for association with uBPA log values across the two examinations. Concentrations of uBPA were positively associated with alcohol intake in participants of baseline and 3rd wave analyses (Table 3). At baseline, uBPA levels were positively associated to carbonate, isotonic and soda drinks consumption (β=0.150, $p$=0.031). This association was not confirmed 9 years later although the number of participants was far smaller. We then tested the association between differences in uBPA concentrations and variations in main covariates across the two analyses; in models adjusted for all main covariates and food variables, changes in daily alcohol consumption were positively associated with differences in uBPA from baseline to follow-up (β=0.104, $p$=0.029; Table 3). In simple correlation analysis the association was confirmed (Spearman correlation=0.174, $p$=0.011): a decrease in alcohol daily intake was associated to a decrease in uBPA concentrations; the same was

true for increased alcohol and uBPA levels. Other food variables in the model were not associated with changes in uBPA concentration, only changes in canned food consumption were nearly significantly associated (Table 3).

***Sensitivity analyses.*** We reran our models of uBPA stability, previously used to assess the correlation between the two uBPA measurements, adjusting also for changes in alcohol, soft drink, canned food, income and BMI across the two analyses. Given the higher number of variables fitted in the sensitivity analysis compared to the initial models, we used the adjusted-$R^2$ coefficient to compare the results with the initial models. Correlation between baseline and 9 year logged uBPA increased in all three regression models, in particular when urine specific gravity was considered (n= 205, r=0.63, β=0.172, 95% C.I. 0.014-0.330; $p$=0.033).

## Discussion

We report levels of uBPA measured in an adult community-dwelling population across a time interval of nine years. Previously, temporal stability of BPA exposure has been studied over short time periods only. In our study we show that 24 hour urine specimens at baseline did show reasonable correlation with spot logged uBPA concentrations 9 years later. To the best of our knowledge, these are the first data on long-term temporal variability of uBPA concentrations, across several years, in a normal adult population.

Two studies investigated uBPA concentrations in a cohort of women before and during pregnancy (17, 18), and indicated that BPA concentrations were variable before and during pregnancy. Childhood levels of exposure over 6 months were reported by Teitelbaum et al. (13), suggesting a reasonable degree of temporal stability. Given the unique features of the cohorts in those studies, conclusions may not be extended to a

general population. Mahalingaiah et al. (14) investigated temporal variability of BPA in 48 subjects over weeks and months and concluded that a single sample showed moderate sensitivity for predicting long-term exposure. Nepomnaschy et al. (19) measured urinary BPA concentration in first-morning samples from 60 premenopausal women over a period of 4 weeks and found that exposures were similar in the short term but became more variable at the end of the 4-weeks period. Other two studies investigated the within-person and within-day variability of uBPA concentrations during a 5/7-days examination in groups of 5 and 8 volunteers (15, 16). Both studies found a considerable variability in the period investigated. Still, no longitudinal studies on a general population have been designed to study the long term variability in uBPA concentrations.

In this study we show that within-subjects uBPA concentrations were significantly correlated. Urinary BPA measurements were correlated in linear regression models adjusted for main covariates as well as models adjusted for indicators of urine dilution such as urinary creatinine and urine specific gravity. Although measurements were significantly correlated, the 218 subjects involved in both analyses had higher geometrical uBPA means at baseline than 3$^{rd}$ wave (Table 2 and 3). The higher values reported at baseline might be explained by an "age effect": age is a clear predictor of uBPA concentration being negatively associated to uBPA in both analyses (see Figure 1). A negative age-uBPA trend was a predictor of uBPA concentrations in two major cross-sectional biomonitoring studies, the NHANES 2003-04/2005-06 surveys (20, 24). Younger subjects generally have higher uBPA concentrations. A cause for the "age effect" is difficult to identify. Potential explanations for differences in uBPA concentrations include changes in routes of exposure (i.e. food and drink ingestion) or changes in BPA metabolism and/or excretion.

Another strong predictor of uBPA exposure in our analysis was alcohol consumption (Table 3). Alcohol and uBPA were strongly positively associated in linear regression models, even when adjusted for main covariates, both at baseline and 3rd wave. This suggests alcohol consumption as a source of BPA exposure, possibly related to storage and packaging of alcoholic drink. BPA has been found leaking into canned alcoholic drink (25) and has been already associated to alcohol consumption in a biomonitoring study (26).

A potential predictor of uBPA concentration in the InChianti study is gender difference. In both analyses, geometrical mean uBPA was higher in male than female (Table 2). At baseline, using 24-hours collection samples, sex was significantly associated with uBPA concentration in models adjusted only for age; no urine dilution correction was performed because of the collection method. Using single spot urine samples (3rd wave), sex was again significantly associated to uBPA concentration in model adjusted for age and major covariates (data not shown). Yet, this association disappeared when adjusting for urine dilution: both in SG adjusted model (Table 3) and creatinine adjusted model (data not shown). Similar to these findings, the NHANES 2003-04/2005-06 surveys reported analogous results (20, 24). It appears that gender difference is a less clear predictor of uBPA, influenced by urine dilution correction.

We investigated other possible predictors of uBPA concentrations such as BMI, annual household income, canned food and soft drink consumption. None of these parameters was a statistically significant predictor of uBPA.

Given the comparison of 24-hours with single-spot urine specimens, it can be claimed that this is a limitation for the study of uBPA temporal stability. While 24-hours urine collection is considered a gold standard for an accurate BPA measure in biomonitoring

study, single-spot urine is regarded as less ideal, given human BPA metabolism and a short biologic half-life (27). It is interesting to note that, uBPA levels measured after single-spot urine collection showed the same trend in age and sex (in SG unadjusted models) distribution as for the 24h analysis.

In conclusion, we have shown for the first time that uBPA concentrations can be relatively stable across lengthy time intervals. Associations between single time point urinary BPA concentrations and health outcomes may be the result of relatively stable long term repeat exposures to BPA. Changing urinary BPA concentrations are positively correlated with changes in alcohol consumption.

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**Table 1.** Demographic characteristics of participants:

| **InChianti Study Characteristics** | **Baseline (1998-00)** | **3rd wave (2007-09)** |
|---|---|---|
| Number of participants (≤75yrs) | 959 | 336 |
| Lost to follow-up (≤75yrs) | | |
| Refused Home Interview | 24 | |
| Emigrated | 13 | |
| Deceased | 13 | |
| Number of participants providing urine specimens (≤75yrs) | 912 | 309 |
| Urine specimens (%) analyzed | 84.3 % | 85.4 % |
| Number of samples <LOD uBPA | 0 | 2 |
| **Participants in both analyses** | | |
| ≤75yrs during the 3rd wave | 218 | |
| Baseline - 3rd wave interval (mean ± SD) | 3339 ± 83 days | |
| Age (mean ± SD) | 48.9 ± 14.3 | 58.2 ± 14.3 |
| Men | 109 | |
| Women | 109 | |
| BMI (mean ± SD) | 26.6 ± 4.1 | 27.2 ± 4.6 |

**Table 2.** Distribution of uBPA concentrations (ng/ml):

| **Characteristics** | | **Geom. Mean (95% C.I.)** | | | **No. of observation** | **%** |
|---|---|---|---|---|---|---|
| **All** | Baseline | 3.87 | (3.57 | 4.19) | 218 | - |
| | 3rd wave | 3.19 | (2.86 | 3.55) | | |
| **Males** | Baseline | 4.13 | (3.66 | 4.66) | 109 | 50.0 |
| | 3rd wave | 3.54 | (3.06 | 4.10) | | |
| **Females** | Baseline | 3.61 | (3.24 | 4.03) | 109 | 50.0 |
| | 3rd wave | 2.86 | (2.44 | 3.37) | | |
| **Age group** | Baseline 21 – 40 yrs | 3.93 | (3.36 | 4.60) | 65 | 29.8 |
| | 3rd wave (*30 – 49 yrs)* | 4.06 | (3.39 | 4.87) | | |
| | Baseline 41 – 55 yrs | 4.21 | (3.67 | 4.81) | 62 | 28.4 |
| | 3rd wave *(50 – 64 yrs)* | 3.66 | (2.99 | 4.49) | | |
| | Baseline 56-65 yrs | 3.61 | (3.17 | 4.12) | 91 | 41.8 |
| | 3rd wave *(65 – 75 yrs)* | 2.44 | 2.05 | 2.89 | | |

**Figure 1.** Age trend in uBPA concentrations at the Baseline (1998-00) and 3rd wave (2007-09) of the study.

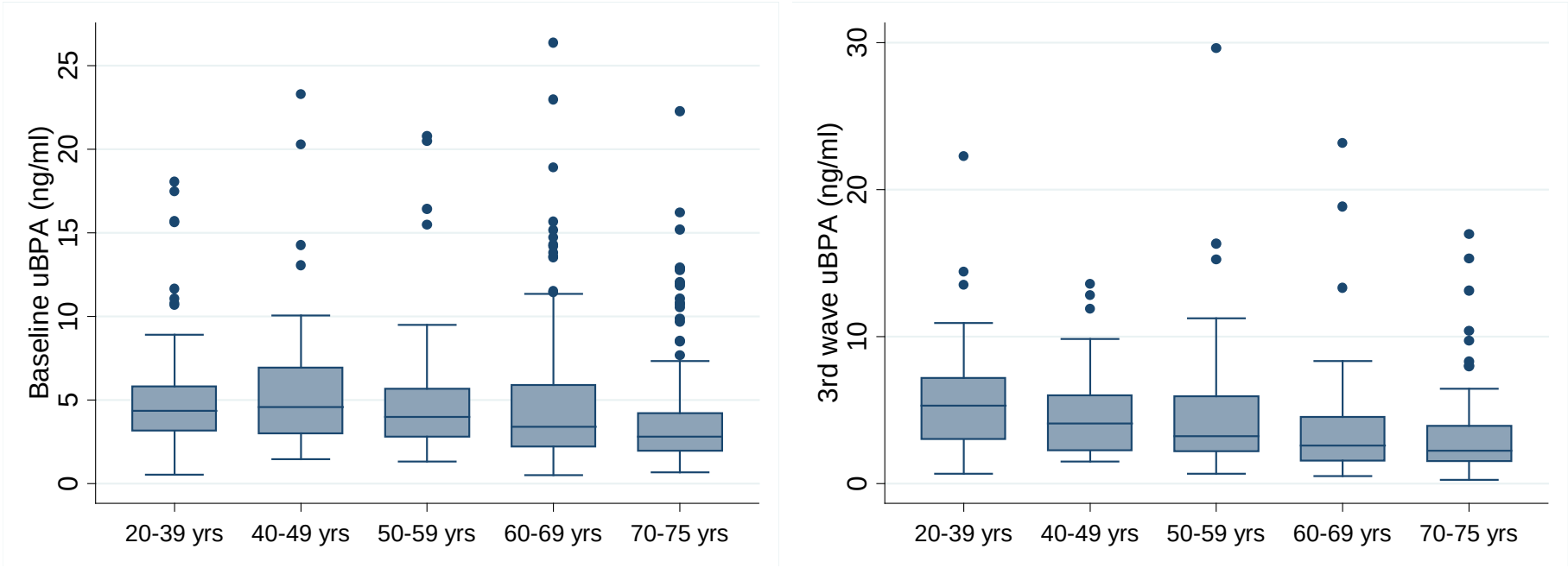

**Figure 2.** Scatter plot of uBPA Baseline log values against 3rd wave SG adjusted log values, with unadjusted linear regression line:

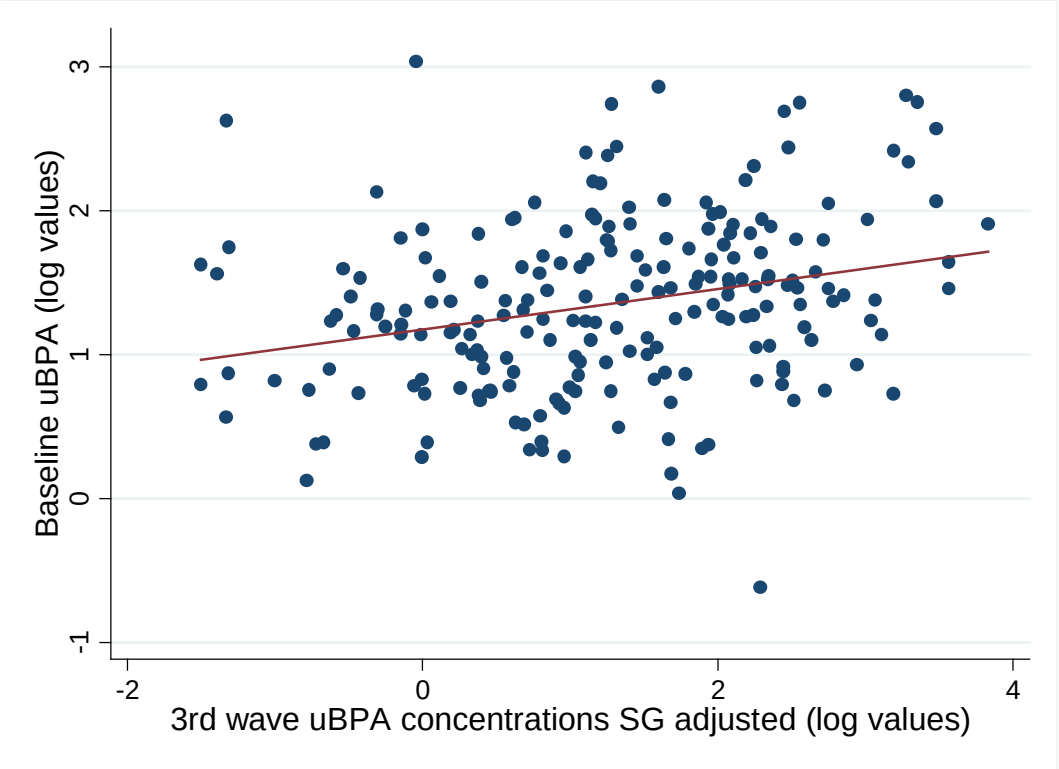

**Table 3.** Association between uBPA concentrations and main covariates at Baseline, 3rd wave, and factors associated with changes in uBPA concentrations. [(a)] Linear regression model in 3rd wave was adjusted for SG measurements. In the third column, log(uBPA 3rd wave)-log(uBPA Baseline) is the dependent variable, the model is adjusted for age, sex and changes in Income, BMI, Alcohol, Canned Food and Soft Drinks consumption. Changes in economic condition were classified as [(b)] worsened income categorization, and [(c)] improved income categorization.

| | | Baseline (1998-00) Association with log(uBPA) | | 3rd wave (2007-09) [a] Association with log(uBPA) | | (3rd wave) – (Baseline) Association with changes in uBPA |
|---|---|---|---|---|---|---|
| Variables | n | beta (95% CI), p-value | n | beta (95% CI), p-value | n | beta (95% CI), p-value |
| Age | 707 | -0.008 (-0.012, -0.005) **p<0.001** | 253 | -0.009 (-0.016, -0.002) **p=0.014** | 209 | |
| Sex (Male v Female) | | -0.093 (-0.199, 0.013) p=0.084 | | 0.006 (-0.178, 0.190) p=0.949 | | -0.099 (-0.335, 0.137) p=0.409 |
| Income | | | | | | |
| Adequate v Barely adequate | | 0.057 (-0.083, 0.198) p=0.425 | | 0.139 (-0.082, 0.359) p=0.217 | | [b] 0.246 (-0.049, 0.542) p=0.101 |
| Adequate v Inadequate | | -0.048 (-0.184, 0.087) p=0.483 | | 0.129 (-0.072, 0.330) p=0.207 | | [c] 0.193 (-0.098, 0.485) p=0.192 |
| BMI | | 0.006 (-0.005, 0.018) p=0.300 | | -0.008 (-0.026, 0.011) p=0.408 | | -0.021 (-0.076, 0.034) p=0.451 |
| Alcohol (freq drinks per day) | | 0.074 (0.040, 0.107) **p<0.001** | | 0.079 (0.021, 0.137) **p=0.008** | | 0.104 (0.011, 0.197) **p=0.029** |
| Canned food (freq per day) | | -0.063 (-0.777, 0.650) p=0.862 | | -0.637 (-2.555, 1.282) p=0.514 | | 1.519 (-0.061, 3.099) p=0.059 |
| Soft drinks (freq per day) | | 0.150 (0.013, 0.286) **p=0.031** | | -0.101 (-0.408, 0.205) p=0.516 | | 0.047 (-0.252, 0.346) p=0.756 |
| $R^2$ (%) | | 9.8 | | 38.0 | | 15.6 |

# 6. Discussion

In the introduction, I outlined how the study of low chronic BPA exposure is still characterised by controversies despite huge scientific efforts. BPA's endocrine disrupting activity has been extensively analyzed in experimental studies and pleiotropic mechanisms of BPA action have been described. Yet, this extensive and sometimes contradictory body of evidence has not been considered conclusive for human risk assessment. Similarly, initial epidemiology investigations have found methodological difficulties in the study of human BPA exposure that have been discussed in section 1.2.3.

The aim of this research was therefore to join both an experimental and an observational approach in order to answer some of the questions, criticisms and limitations of previous studies.

Here, I present the levels of human exposure to BPA measured for the first time in a European representative population. These results indicate a widespread BPA exposure with levels of detection nearing the totality both at baseline and nine years later, in the 3$^{rd}$ wave (*Chapter 2*, *3* and *5*). Moreover, accurate uBPA concentrations measured in 24 hours urine samples were associated with changes in hormones levels, testosterone and SBHG, indicating a physiological effect of BPA action (*Chapter 2*). Further, BPA bioactivity was investigated at molecular levels. Evidences from *in vivo* and *in vitro* studies showed how BPA can modulate the gene expression of the hormone receptors ERβ and ERRα (*Chapter 3* and *4*). Finally, I provide the first results on the exposure to BPA in adult subjects analysed over a period of nine years and report on the pattern of stability over this time frame (*Chapter 5*).

These results are important because, taken together, they suggest that BPA is bioactive

in the human body and suggest possible mechanisms of action both at the molecular and physiological level. In addition, this research provides useful indications for the design of future epidemiological studies.

*6.1 An integrated approach in the study of Bisphenol A.*

As discussed in section 1.2.3, human exposure to BPA has been recently associated with metabolic disorders like obesity, diabetes and, ultimately, with cardiovascular disease (CVD). In particular, an increased risk of cardiovascular disease has been repeatedly associated in adults with exposure to BPA.

Epidemiological findings are able to associate level of exposure, uBPA, to a disease outcome, CVD, but with no indication on the causal relationship between the two. In other words, there is a "black box", a lack of information on how BPA could cause, at biological level, the onset of the disease.

Four main criticisms have been raised over the plausibility of the association with CVD found in the cross-sectional studies (see section 1.2.3). The main criticism has been raised because of this black box: is BPA bioactive at the concentrations measured in the human body? What is the molecular mechanism of BPA action in the human body? These are clear limitations for standard epidemiology studies.

In order to answer these questions and move forward in the study of BPA it became apparent the need for a more "holistic" approach to the problem.

A possible way to unravel the black boxes is described by the meet-in-the-middle (MITM) principle (Vineis et al. 2013): in order to confirm the plausibility of the link between exposure and disease it is fundamental to determine intermediate biomarkers

that could link the two.

*The reasoning is in three steps. The first step of this approach consists in the investigation of the association between exposure and disease. The next step consists in the study of the relationship between (biomarkers of) exposure and intermediate omics biomarkers of early effects; and third, the relation between the disease outcome and intermediate omics biomarkers is assessed. The MITM stipulates that the causal nature of an association is reinforced if it is found in all three steps (Vineis et al. 2013)* .

This research looked for intermediate biomarkers at different level of biological organisation in order to contribute addressing some of the outstanding controversies of this field.

### *6.1.1 Molecular findings.*

As listed in section 1.3 and in Figure 1.2, of particular importance was to study the hypothesis that BPA is bioactive in the human body. The expression of ERβ and ERRα were both shown to be influenced by exposure to BPA both in *in vivo* and *in vitro* analyses. The advantages of using an integrated approach were multiple.

First, ERRα emerged as a novel target of BPA hormone modulating capacity: while indications of BPA interaction with ERβ have been extensively reported in animal and cell studies (see section 1.2.2), ERRα did not emerge from the literature as a possible target of BPA action.

Second, the validation study, carried out on a human leukemic T-cell line, investigated the *in vivo* hypothesis in laboratory settings where potentially confounding variables were controlled. The validation of the original findings using a human blood cell line diminishes the possibility that the results in the *in vivo* study were masked by confounding factors.

Moreover, the identification of ERRα is important not only because its expression is highly associated with high levels of uBPA. In fact, ERRα appears to be a promising

intermediate biomarker linked also with metabolic diseases and CVD.

ERRα activation is modulated by a possible interplay with the transcriptional PPARγ co-activator (PGC-1α) (Willy et al. 2004). ERRα has been described as pivotal in cardiac functions and adaptation to pressure overload, and is believed to exert protective effects against stressors known to cause heart failure (Dufour et al. 2007; Huss et al. 2007). Also, it has been suggested that the modulation of ERRα and ERRγ activities could be used to manage cardiomyopathies (Huss et al. 2007). ERRα is implicated in the production and transportation of ATP across the mitochondrial membranes as well as $Ca^{2+}$ handling (Dufour et al. 2007). Interestingly, recent studies have shown that BPA can induce arrhythmia in murine models via alteration of $Ca^{2+}$ handling (Asano et al. 2010; Yan et al. 2011).

Moreover, the modulation of the transcriptional circuit ERRα /PGC-1α has been suggested as a possible target in therapies for type 2 diabetes (Handschin and Mootha 2005) as well as having a role in the onset of obesity (Casals-Casas and Desvergne 2011; vom Saal et al. 2012). These results taken together are indicating a possible molecular mechanism of BPA in metabolic disruption.

*6.1.2 Physiological findings.*

In addition to the molecular evidence, this research provides indications also on the physiological effects of the exposure to BPA, and in particular on possible changes on testosterone blood levels in men.

This is important because the effect at the physiological level confirms the bioactivity of BPA. Moreover, there could be a link between the occurrence of CVD and higher testosterone levels. Although just in woman, higher levels of testosterone have been linked to increased risk of incident coronary events (Laughlin et al. 2010). In a follow-

up study of nearly 13 years, the authors found increased risk of CVD events when total and bioavailable testosterone levels were outside an optimal range.

Furthermore, the association between BPA exposure and testosterone levels could also have repercussions on another aspect of the EDC research. In recent years, the so-called estrogen hypothesis (Sharpe and Skakkebaek 1993) proposed that the steady decline in sperm quality and the high occurrence of male reproductive disorders, such as hypospadias, observed in Europe in the last 30-50 years could be due to male exposure to estrogen-like compound like BPA.

More recently, this theory has been expanded to suggest that male *in utero* exposure to oestrogen and anti-androgen compounds could be responsible of several reproductive problems that could then lead not only to sperm quality decrease but also to testicular cancer (TC) (Skakkebaek et al. 2001). Different studies have shown an increase in the incidence of TC in the last 40 years with substantial differences among countries (Huyghe et al. 2003). In particular in northern Europe, surprising differences in incidence rates were seen between neighbouring countries (Finland 2.5/100,000 cases versus Denmark 9.2/100,000). Environmental hypotheses with a key role of endocrine disrupters have been put forward in order to explain such a striking geographical imbalance.

Skakkebaek and colleagues hypothesized that TC could originate from primordial germ cells with altered differentiation *in utero* due to exposure to EDC which would also result in an impaired testosterone production. These findings confirm the ability of BPA to affect the level of testosterone in the male body although more research is needed.

The link between the findings at the molecular and physiological level is less clear to identify. A recent study investigated the effect of BPA on testosterone production in Leydig cells (N'Tumba-Byn et al. 2012); the authors suggested that BPA could act

through non-classical estrogen receptors signalling pathway. They suggested that ERRγ or an unknown ligand of ERRγ could be responsible of the mechanism of action of BPA in regulating testosterone production.

*6.1.3 Methodological issues investigated.*

Another controversy that this research investigated is the stability of BPA exposure over lengthy periods. Given the results of cross-sectional studies, one of the main criticisms of these studies was the lack of indications over the stability of chronic exposure to BPA. From a methodological point of view this research is important because we now have a better understanding of BPA stability over a long period. Results indicate that a single measure of uBPA concentrations showed moderate sensitivity for predicting long term exposure (*Chapter 5*). This adds to the plausibility that reported associations with health outcomes may be causal.

There were other controversies listed in section 1.2.3 regarding (a) the possibility of a reverse interpretation of the results, and (b) the risk of misclassification due to the self-reported diagnoses of cardiovascular disease. These have been widely addressed by a recent "nested case-control" epidemiology study (Melzer et al. 2012a) where uBPA concentrations were measured nearly 11 years before the participants' first medical diagnose of cardiovascular disease. Given the study design, both (a) and (b) could be ruled out as possible explanations for the results.

*6.2 Limitations and confounding factors.*

This research presents some limitations that should be considered when interpreting the results. In particular, given the contradictory evidence in the literature, the testosterone findings need to be replicated in a representative population study. Unfortunately, no

measure of sex hormone concentrations were performed during the 3rd wave of the InChianti study so it was not possible to reproduce the same analyses carried out at the baseline of the study and reported in chapter 2.

Moreover, the controversy over the stability of BPA exposure during a significant time window cannot be considered resolved. Measurements are still needed to understand the value of a single measurement associated to diseases with long latency periods. Ideally, the two other follow-ups in the InChianti study (2001-03 and 2004-2006) could provide valuable indications and a much more detailed description of BPA stability over nine years. Unfortunately, it was not possible to analyze samples from these time-points because of costs and practicalities.

This project focussed on the effect of human environmental exposure to a single compound, a single EDC. However, we acknowledge the fact that there is a number of compounds to which we are exposed on a daily basis that could have estrogen-like properties. The list is long and in continuous expansion. Not only man-made compounds, natural estrogens can be found in cow's milk and meat: modern dairy cows are usually pregnant and continue to lactate during the latter half of pregnancy, when the concentration of estrogens in blood, and hence in milk, increase (Ganmaa and Sato 2005). Meat and milk consumption have been linked to hormone induced cancer such as ovarian and breast cancer.

The combined effect of multiple exposures is certainly an issue to consider when interpreting the results of this research. A combined exposure could have a confounding effect on the study of a single compound, like in this case BPA. It could be argue that the confounding effect could operate in two different ways. On the one hand, this could cause a form of white noise when measuring the effect of BPA *in vivo*: exposure to natural estrogen, for instance, could make more difficult the detection of a molecular

biomarker of BPA action. For this reason, it was important to test the effect of BPA exposure *in vitro* in order to rule out the effect of any confounder.

On the other hand, it could be argued that isolating the effect of a single compound from a mixture of estrogen-like compound is not possible. In this case, the study of the exposure to a single compound can be considered a simpler scenario given the complexity of conducting a cumulative risk assessment. However, the risk of BPA effects being confounded by the exposure to other estrogen compounds is limited unless we consider BPA exposure to be highly correlated with the other compounds.

### *6.3 Regulation: Politics of Bisphenol A.*

As discussed in the introduction, BPA has been extensively studied in the last 15 years. Scientific knowledge has rapidly built and started to reach the public interest until the point when in 2008, BPA was making headlines in major national newspapers both in the US and Europe.

In the same year, the Canadian government announced its decision to declare BPA toxic. Health Canadian Minister announced Canada's intent to ban the import, sale, and advertisement of polycarbonate baby bottles containing Bisphenol A due to safety concerns (Vogel 2009).

From 2009 and 2011 a number of European countries started considering the ban of BPA from infant product. In April 2011, the European Union decided that BPA was "*not to be used for the manufacture of polycarbonate infant feeding bottles*" (EU 2011).

However, public outcry and mounting concern had already pushed retailers and producers to meet growing demands for alternatives to BPA-based polycarbonate baby and water bottles. BPA-free baby bottle started to appear in the market well before the 2011.

When in 2012 the US F.D.A. decided to follow a similar path and to ban BPA from infant product, manufacturers had already stopped using the chemical and baby bottles containing BPA were already out of the market.

Interestingly, none of these decisions were the result of indications from the US or European regulatory agencies. As described in section 1.2.2, the US EPA established in 1998 the BPA human TDI on the basis of results from carcinogenic studies on rats. It quickly became clear how the old paradigms of toxicology and carcinogenic studies could not be applied to the study of EDCs.

Scientists have shown in the last 20 years that a new scientific paradigm should be used to define chemical safety. New concepts such as low-dose research and non-monotonic dose response are opposing the traditional safety standards based on threshold-dose models or high-dose toxicity tests. Low-dose BPA studies explored new endpoints and focused on measure of organizational and functional changes as indicators of disease risk. They also looked at the relationship between timing of exposure and measured effect.

All these concepts have been cautiously considered by the regulatory agencies who were asked to accept a large scientific paradigm shift to carry out the BPA risk assessment. As described in section 1.2.3, successive reviews from the US NTP of the scientific literature did not found convincing evidence and the TDI was upheld (Chapin et al. 2008; Melnick et al. 2002). Yet, many more studies and different endpoint were included in the second review and low-dose research is no longer on the margins of accepted scientific thought.

Similarly, the European Food Safety Authority, the EU equivalent of the American NTP, was asked to review the epidemiological literature on BPA and CVD (EFSA Panel on Food Contact Materials 2010). They concluded that the study design "*does not*

*allow establishment of a causal relationship between BPA exposure and health effects*" and highlighted the "*lack of a common clearly defined mode of action of BPA at low doses*". Results from this research may help addressing the question raised by the EFSA Panel.

## 7. Conclusions

Different indications can be drawn from this research. First of all, analyses from the InChianti study confirms the initial hypothesis that BPA is a widespread, ubiquitous compound to which virtually all persons in industrialized countries are exposed to. Moreover, this research strongly suggests that BPA can be bioactive in the human body at environmentally relevant concentrations. This consideration is motivated by physiological and molecular findings: BPA was associated with changes in testosterone levels and in estrogenic gene expression targets in men. Further, laboratory evidence has confirmed ERRα and ERβ as targets of BPA action in human blood cells. In addition, this research provides moderate indication of a long term stability of human BPA exposure with measurements from samples collected nine years apart. These results are supportive of previous epidemiological findings in which BPA exposure was associated with metabolic syndromes. Further studies are needed to understand the potential of ERRα modulation to determine the onset of health diseases, in particular examining BPA exposure in a wider range of estrogen-regulated target tissues.

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